SQUID PROTEIN CHARACTERISTICS AND THEIR POTENTIAL INDUSTRIAL APPLICATIONS

CLAUDIA LIZETH MURRIETA-MARTÍNEZ, VÍCTOR MANUEL OCAÑO-HIGUERA, GUADALUPE MIROSLAVA SUÁREZ-JIMÉNEZ AND ENRIQUE MÁRQUEZ RÍOS

SUMMARY

The global increase in population has caused a rise in the food demand and seafood is no exception. This has led to an overexploitation of commonly consumed species, and new species have been proposed to satisfy current demands. One such species is the giant squid. This species has a great potential due to its intrinsic characteristics. This fishing resource is one of the most important in Mexico, but only 10-20% of the catch is consumed domestically. The rest is exported to Asian countries, with minimal added value. In this sense, integral uses of this resource must be considered, as the mantle, fins, head and viscera can be used for different purposes. In this review, we summarize the major characteristics of squid proteins and their application as food ingredients, in aquaculture and in other practical applications, as well as what has been and is being done in the industry.

The giant squid (*Dosidicus gigas*) is an abundant resource found in the pelagic zone of the Eastern Pacific and is the largest and most abundant squid species from Chile to the Northwest coast of the United States (Nigmatullin *et al*., 2001). In Mexico, it is abundant in the Gulf of California (Markaida, 2005).

This species has many outstanding characteristics that are important to consumers, including high yields after gutting because the viscera only account for 10%, easy evisceration, lean white flesh without bones or scales, and low cost (Campo-Deaño *et al*., 2009). These features make the giant squid an attractive cephalopod species for product development (Encinas-Arzate *et al*., 2014). However, the local population prefers other species such as shrimp, shark (*cazón*), ray, and other fish, and thus giant squid is highly underutilized, with only 10-20% of the catch being consumed in Mexico. The rest is exported fresh, fresh-frozen or cooked-frozen to other countries, mainly to Asian markets, with minimum processing and very low added value. It is therefore important to add value to this resource, so as to gain a better perspective of consumption and better prices. Recent efforts have developed value-added products in which jumbo squid could be used (Campo-Deaño *et al*., 2009). The characteristics of the squid's mantle (white, lean, no bones or scales) make it a good alternative for protein concentrates, which could be used for manufacturing surimi. The mantle and by-products could also be used to obtain protein hydrolysates, collagen and pigments, while the viscera are rich in enzymes and lipids. Numerous studies have been made of these by-products to reach a more integrated approach to this resource, but more research is still necessary, especially in the development of value-added products. The aim of this review is to summarize some of the studies leading to solutions for a better use of squid proteins and by-products regarding the current state of the industry.

KEYWORDS: / *Dosidicus gigas* / Gelation / Giant Squid / Functionality / Proteins /

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Claudia Lizeth Murrieta Martínez. Food Chemist, M.Sc. and doctoral student in Food Science, Universidad de Sonora (UniSon), Mexico.

Víctor Manuel Ocaño Higuera. Doctor in Food Science, Centro de Investigación en Alimentación y Desarrollo (CIAD), México. Professor, UniSon, Mexico.

Guadalupe Miroslava Suárez Jiménez. Doctor in Food Science, UniSon, Mexico. Professor, UniSon, Mexico.

Enrique Márquez Ríos. Doctor in Food Science, CIAD, Mexico. Professor, UniSon, Mexico. Address: Departamento de Investigación y Posgrado en Alimentos, UniSon. Rosales y Niños Héroes S/N. P.O. Box 1819. Hermosillo, Sonora 83000, Mexico. e-mail: emarquez@guayacan.uson.mx

Giant Squid Generalities

Squid muscle is a source of high-quality protein because it is readily digestible and possesses all of the essential amino acids. Appropriate processing of jumbo squid muscle can result in enriched products. However, attempts to process jumbo squid have failed because of scarce knowledge of its physiological and intrinsic characteristics (De La Fuente-Betancourt *et al.*, 2009). The high proteolytic activity in the mantle (Konno *et al.*, 2003) results in functional differences of protein concentrates made of squid mantle compared to other marine species. However, most studies reporting a rapid quality loss worked with squid that was frequently subjected to poor post-capture management; for example, specimens left in piles without evisceration or cooling treatment for several hours, sometimes reaching temperatures up to 35ºC (Márquez-Ríos *et al.*, 2007).

Mantle composition

The squid mantle is composed of five different tissue layers (Martínez *et al.*, 2000), where bands are divided into two types: circumferential, composed of fibers running around the entire circumference of the mantle cone, and radial, composed of fibers that connect two tunics of connective tissue. The exterior tunic is made of collagen fiber layers adjacent to an external layer of connective tissue fibers, which are located under the skin (Otwell and Giddings, 1980).

The general chemical compositions of the mantle, fins and tentacles are similar to those of non-fat fishes. However, squid muscle fibers and connective tissue are stronger than those of fish muscle and their arrangement is also very different (Stanley and Hultin, 1984; Sugiyama *et al.*, 1989). It contains a different class of myofibrillar proteins that are more water soluble and have a different organization, being less susceptible to freezing and more prone to thermal denaturation (Kolodziejska *et al.*, 1999).

Protein composition

Proteins from squid mantle muscle differ from those of marine vertebrates. The myofibrillar fraction constitutes ~75-85% of it, with myosin as its major component followed by actin and paramyosin, the latter being particularly common in marine invertebrates and can represent up to 25% of themyofibrillar proteins (Sikorski and Kolodziejska, 1986; Cortez-Ruiz *et al.*, 2008). Sarcoplasmic proteins represent ~15% of the total, and has endogenous proteolytic activity that could be responsible for the high rate of autohydrolysis (Arias-Moscoso *et al.*, 2014; Sánchez-Sánchez *et al.*, 2014). An important difference in relation to other marine organisms is the higher stromal fraction, composed primarily of connective tissue and representing 11% of all proteins (in fish muscle it amounts to 2-3%). These differences are primarily related to the physiological needs of the jumbo squid, such as their high energy movements needed for locomotion (Macgillivray *et al.*, 1999; Kier and Curtin, 2002; Kier and Thompson, 2003). To propel itself through the water using jet propulsion, an elastic but also strong mantle is necessary, which results in the fiber crosslinking and the high amounts of collagen found in the skin and mantle.

Protein Characteristics and Opportunities

High proteolytic endogenous activity

Cephalopods typically have a high level of proteolytic activity, higher than most fish species (Stanley and Hultin 1984; Kolodziejska *et al.*, 1987; Hurtado *et al.*, 1999). For this reason *D. gigas* muscle undergoes intense proteolysis immediately after capture (Nagashima *et al.*, 1992; Gómez-Guillén *et al.*, 1996). However, the high endogenous enzymatic activity reported in giant squid muscle (Ayensa *et al.*, 2002; Ezquerra-Brauer *et al.,* 2002; Gómez-Guillén *et al.,* 2002; Ruiz-Capillas *et al.,* 2003) could be taken advantage of in order to recover soluble proteins from squid by-product by auto hydrolysis, which could be used for aquaculture feed.

Obtention of auto hydrolysates is advantageous since one of the most expensive ingredients in feed production is protein, and different protein sources are used to reduce costs. Protein hydrolysates possess healthy and nutraceutical properties and have been used as food supplements (Haard, 2001). This would be particularly valuable in a starter diet for fish larvae since they are unable to digest and assimilate nutrients efficiently (Lian *et al.,* 2005). Sánchez-Sánchez *et al.* (2014) compared the hydrolysates of head, tentacles and skin from jumbo squid by-products, obtained by auto-hydrolysis (55ºC, pH 5.0) and a chemical-enzymatic process (45ºC, pH 2.5 and pepsin). After 90min, 18-30kDa proteins were observed in both processes, indicating that endogenous enzymatic activity in giant squid is enough to obtain hydrolysates with good characteristics. This was confirmed by Arias-Moscoso *et al.* (2014), who obtained hydrolysates of skin, head and fins jumbo squid, at two different pH values (5.0 and 7.0) by means of its endogenous proteases. Both treatments exhibited similar degradation patterns, with <45kDa proteins observed after 120min of hydrolysis. Endogenous proteases in squid by-products can produce hydrolysates with useful properties, resulting in a higher yield at pH 7.0. It was hypothesized that this type of hydrolysate could be used in shrimp feed due to its characteristics (González-Félix *et al.,* 2014). The authors evaluated the use of squid hydrolysates obtained by acid-enzymatic hydrolysis and auto hydrolysis as ingredients in shrimp (*Litopenaeus vannamei*) diets at 2.5 and 5.0% dry weight. There was significantly higher crude protein in shrimp muscles with the highest hydrolysate levels (5%). No effect on growth or survival was observed, indicating that they could be utilized to partially replace sardine fishmeal in aquafeed. The authors concluded that hydrolysates from jumbo squid by-products can be potentially utilized in fishmeal for the aquafeeds industry.

Squid hydrolysates can also be used as organic fertilizers, which are naturally low in nitrogen (N) and rely on microbial activity to mineralize organic N into plant-available forms. Hydrolysates, can improve their quality because N is released more slowly from organic sources over longer periods of time as compared to synthetic fertilizers (Fetter *et al.*, 2013). Organic fertilizers have the potential to increase soil fertility and soil organic matter content (SOM) on the long term (Booze-Daniels and Schmidt, 1997; Nardi *et al.*, 2004). The conversion of squid by-products into organic fertilizer may present a solution to problems with high disposal costs for squid processors. The high N content (75% dw) associated with squid by-products, suggests their potential to develop a marketable product from waste (Fetter *et al.*, 2013).

Peña-Cortés *et al.* (2010) studied the effect of the hydrolysate obtained from squid waste using alcalase as a proteolytic enzyme (55ºC, pH 7.5). The hydrolysate was applied through irrigation because it appeared to have a negative effect on plants vegetative tissues when applied directly. The use of hydrolyzed waste irrigation solutions caused an increment in the foliar diameter of treated plants, increasing whith higher digestion time and hydrolysate concentration. Seed yields were also significantly different in treated plants.

Fetter *et al.* (2013) prepared granular and liquid squid fertilizer from head, fin, viscera, mantles and tentacles to evaluate their effectiveness compared with synthetic fertilizer on soil fertility and turfgrass quality. Organic fertilizers are often presumed to increase soil PO_4 concentrations (Wright *et al*., 2008), which can increase the risk of environmental pollution (Ginting *et al*., 2003). However, this was not the case in the present study. An important parameter is microbial activity, closely related to soil fertility through mineralization of organic forms of nutrients from SOM and dead microbial biomass into plant-available inorganic forms of nutrients (Frankenberger and Dick, 1983). Both liquid formulations produced significantly higher microbial activity than the granular products, probably as a result of the decreased time and lower energy required for microbes to break them down. Also, squid hydrolysates produced higher microbial activity values compared with the synthetic formulations (Fetter *et al*., 2013).

Regarding turf quality, it displayed uniformity and density along with high clipping production, which reflects sufficient N availability (Turgeon, 2012). When quality was examined over the entire study period, there were no significant differences among the four products (synthetic and squid hydrolysates, both liquid and granular). Thus, the use of squid-based products offer a turf quality comparable to synthetic fertilizers. Overall data suggest that squid hydrolysate can be effective as an organic fertilizer applied to turfgrass, either in liquid or granular state. It consistently provided high-quality, and uniform turf when compared with synthetic fertilizers applied at the same level (Fetter *et al*., 2013).

Thermal instability

Thermal stability is essentially the resistance of the protein molecule to unfolding as a result of any thermal treatment (Bernal *et al*., 1987). Myofibrillar proteins in the muscles of several species form aggregates between 40-60ºC, but in giant squid unfolding begins at 30-32ºC, followed by protein association at 45-50ºC (Kristinsson and Rasco, 2000; Tornberg, 2005; Tolano-Villaverde *et al*., 2013). This behavior is unexpected, because giant squid dwells in warm waters and their proteins should have higher thermal stability.

This instability could be related to the low content of sulfhydryl groups present in the myosin molecule, which have been reported at ~0.9mol/10^5g of protein, whereas most fish species contain around ~4.8mol/10^5g of protein. Sulfhydryl groups confer stability, so lower values would make the molecule less stable (Murrieta-Martínez *et al*., 2015). Myosin is the main component of the myofibrillar fraction, which makes up the majority of muscular proteins. Nevertheless, the instability may also be related to a high autolytic activity. Squid mantle muscle contains endogenous metalloproteinases that selectively degrade myosin molecules into heavy meromyosin (HMM) and light meromyosin (LMM) (Yoshioka *et al*., 2005).

However, even when marine proteins are known for their thermal instability, squid proteins in particular have shown a high stability under freezing, which can compensate the behavior previously reported.

High solubility

Functional properties of seafood muscle are highly related to protein solubility (Valencia-Pérez *et al*., 2008). All publications on the solubility of giant squid proteins (Borderias and Montero, 1985; Sato *et al*., 1991; Ando *et al*., 1999, 2001; Enzinas-Arzate *et al*., 2014) have concluded that muscular proteins in squid are highly soluble and, thus, conventional washing processes used to produce surimi from fish are not a viable alternative for obtaining protein concentrates from this species (Sánchez-Alonso *et al*., 2007). Even when most proteases and all compounds producing an undesirable odor and bitter taste are removed, a large portion of the myofibrillar proteins are solubilized and washed away, reducing the total protein yield (Palafox *et al*., 2009).

This behavior has been reported by Rocha-Estrada *et al.* (2010), who evaluated several properties from squid mantle and fin proteins under operational variables, particularly pH and ionic strength. They found that solubility was a requisite for useful protein properties. For example, a homogenate with highly soluble protein is expected to have a high foaming capacity. The SDS-PAGE pattern showed the presence of the myosin heavy chain (MHC) in the sarcoplasmic fraction, indicating high solubility of squid myofibril proteins even at low ionic strength. This phenomenon is of particular interest for processes that involve washing with water, as in surimi production. If squid muscle is washed with water to isolate myofibril proteins, the MHC fibers could be lost by solubilization.

Galvez-Rongel *et al.* (2014) compared acidic (AcPC), alkaline (AkPC), isoelectric (IPC) and neutral (NPC) protein concentrates and reported that during protein fractionation at low ionic strength (I= 0.05), the soluble protein fraction was 4.98 ±0.09%, 7.7 ±0.2% and 5.83 ±0.84% for AcPC, IPC and AkPC, respectively. However, even though most of the sarcoplasmic fraction was removed, NPC still contained a high amount of soluble protein with values of 20.5 ±1.5%. This confirms the high solubility of squid mantle protein, previously documented in other studies (Cortes-Ruiz *et al*., 2008; Dihort-Garcia *et al*., 2011).

Encinas-Arzate *et al.* (2014) evaluated washing with different ionic strengths (I= 0.0, 0.1 and 0.3) and reported high solubility of myofibrillar proteins from squid muscle at low ionic strengths. Similar results were reported by Gomez-Guillen *et al.* (1996), who studied the solubility of squid mantle protein (*D. gigas*) as a function of temperature and ionic strength. These researchers detected the presence of electrophoretic bands corresponding to actin, tropomyosin and low molecular weight proteins at 0.05M NaCl. They concluded that the removal of sarcoplasmic proteins by increasing ionic strength also gradually removed myofibrillar proteins because of their high solubility.

Stability under freezing

In cephalopods, the myofibrillar proteins are highly resistant to freeze-induced denaturation (Moral *et al*., 1983). Gómez-Guillén *et al.* (2003) determined changes in the functional and chemical properties of squid muscle proteins during 10 days of chilled (2ºC) and 30 days of frozen (-20ºC) storage. Functionality, apparent viscosity and extractability of proteins were not affected; even thermal behavior remained relatively stable. The SDS-PAGE profile showed MHC degradation and its further disappearance, but paramyosin and actin remained stable and were unaffected during the entire study, confirming the freezing stability previously reported.

This property, as stated above, may prove useful for the development of value-added products such as protein concentrates, imitation of restructured fish fillets and emulsified-gel products where preserving the gelling capacity of the muscle is essential even after the freeze-thaw processes. Without the use of cryoprotectants, most muscles lose functionality when frozen because of protein denaturation and/or aggregation (García-Sánchez *et al*., 2015), but this is not the case for giant squid muscle. Additionally, as raw material, giant squid would be an ideal target for these types of products due to its high post-process yield, low fat content, white muscle, scaleless, boneless and good organoleptic characteristics (Campo-Deaño *et al.*, 2009).

García-Sánchez *et al.* (2015) noted that the gelling capacity of the jumbo squid muscle was maintained even after frozen storage without the use of cryoprotectants. They evaluated the effect of freezing (-20ºC) and thawing (4ºC) on protein denaturation and gelling capacity the *D. gigas* mantle muscle. In a differential scanning calorimetry study, they observed similar patterns in T_{max} and enthalpy and no denaturation in any of the thermograms, suggesting a high stability of squid muscle proteins. Increased surface hydrophbicity (SoANS) values suggested that proteins had unfolded due to freezing, but increases in enthalpy suggested protein aggregation. They hypothesized that the interactions formed among proteins were reversible. Finally, the quality of gels as determined by a folding test, water holding capacity (WHC) and texture profile analysis (TPA) were not affected by any treatment. With this evidence, they concluded that freezing for up to 30 days does not affect squid muscle proteins or their gelling capacity, and squid muscle should be prioritized over other muscle types as a raw material for value-added products.

This resistance to freezing can be explained by the presence of paramyosin (Iguchi *et al.*, 1981), a protein present only in marine species that decreases the rate of protein denaturation. Paramyosin is found in greater amounts in squid, representing ~14% of the myofibrillar fraction. This could explain the freeze resistance reported in squid proteins.

However, gels made from giant squid (*D. gigas*) have lower gel strength than the ones made from other fish species (Sánchez-Alonso *et al.*, 2007). Additionally, application of the traditional surimi process to squid results in a concentrate with reduced functional-technological quality and low yield (Matsumoto, 1958). The acidic/alkaline solubilization process developed by Hultin and Kelleher (1999, 2000) is a better option to obtain protein concentrates from this species. Under acidic conditions (pH= 3), the concentrates display high gel strength, elasticity, and cohesiveness (Cortés-Ruiz *et al.*, 2008) while alkaline dissolution (pH= 10) promotes autolysis of the myosin heavy chain and low water retention, resulting in better protein solubility and similar protein recovery as under acidic conditions. On the other hand, the gel-forming capacity was improved over traditional methods (Dihort-García *et al.*, 2011).

Another option is the use of differential ionic strength process, during the preparation of protein concentrates to discard sarcoplasmic proteins. However, myofibrillar proteins can support low levels of sarcoplasmic proteins without affecting the resulting gel properties. Encinas-Arzate *et al.* (2014) used NaCl solutions of different ionic strengths (I= 0.0, 0.1, and 0.3) to obtain three protein concentrates. They found that hardness increased when I increased, as well as S-S bond formation, during thermal gelation. This indicates that removal of sarcoplasmic proteins as a function of I results in better quality gels.

Jumbo squid proteins can also be an important protein source incorporated into food products, either as a raw material or as protein supplement (Palafox *et al.*, 2009). An example of this is the use of squid protein as a flour additive. Similar studies have been carried out by Ramírez-Suarez *et al.* (2012), who added lyophilized jumbo squid fin (JSF) and mantle (JSM) to commercial wheat flour and evaluated the effects on the resulting dough and bread properties. The addition of JSF and JSM during dough production increased resistance to mixing, likely as a consequence of the strengthening of the protein network by the animal protein. However, stronger dough does not necessarily produce a larger bread loaf; a balance between dough strength and extensibility is required (Wrigley *et al.*, 2005). The most relevant characteristic was the bread loaf protein content, which was significantly different from the control. The control samples contained 92.8 ±6.0$g{\cdot}kg^{-1}$ protein, while the samples with squid muscle, either JSF or JSM at 50g/kg, had higher contents of ~105.8 ±3.4$g{\cdot}kg^{-1}$, which represents an important nutritional advantage. Furthermore, the sensory analysis showed acceptable results for this type of bread when there was an addition of 25$g{\cdot}kg^{-1}$ of JSF or JSM (protein content was still higher than the control, 98.8 ±1.0$g{\cdot}kg^{-1}$), being a good option for a value-added product (Ramírez-Suarez *et al.*, 2012). Matrel Foods S.A.C. has already developed a bread enriched with squid flour as the base, but they only worked with the mantle region**.** This new product is distinguished by its high protein content (86% *vs* 8% in common bread), level of unsaturated fat, and source of ω3 and ω6 fatty acids (Anonymous, 2007).

Squid flour (called CPP-Lamolina) has been produced since 2001 by the Universidad Nacional Agraria La Molina, Peru, and it is used in several enriched foods for human consumption. This concentrate comes from the mantle region and has a high protein content of ~85%, whereas similar products such as milk powder and eggs only contain up to 26% and 12.5%, respectively. It is also shelf-stable and contains ω3 fatty acids. CPP-Lamolina can be incorporated as an ingredient without impacting the appearance, smell or flavor of food products and is currently being added to products such as enriched bread, noodles, chocolate coated flakes, and wheat flour (Roldán-Acero, 2007).

Conclusions

Value-added products have received great attention in recent years. One of the most prominent uses of squid proteins is the production of protein concentrates from the mantle, fins or tentacles. Thus, there are many uses for this organism, like a protein-rich additive for bread making or the obtention of protein hydrolysates for aquaculture feed and for the elaboration of organic fertilizers. Other possibilities (not reviewed in this work) include the characterization of fatty acids from the viscera, chitosan from the pen, and pigment recovery. This review presented the major characteristics of squid proteins and some applications for them. More uses of this resource must be considered, and the mantle, fins, head and viscera should be used for different purposes.

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CARACTERÍSTICAS DE LAS PROTEÍNAS DE CALAMAR Y SU POTENCIAL APLICACIÓN INDUSTRIAL

Claudia Lizeth Murrieta-Martínez, Víctor Manuel Ocaño-Higuera, Guadalupe Miroslava Suárez-Jiménez y Enrique Márquez Ríos

RESUMEN

El aumento global de la población ha provocado un aumento en la demanda de alimentos y los productos marinos no son la excepción. Esto ha llevado a una sobreexplotación de las especies de consumo habitual, por lo que han sido propuestas nuevas especies para satisfacer las demandas actuales. Una de estas especies es el calamar gigante, cuyas características le otorgan un gran potencial. Este recurso pesquero es uno de los más importantes de México, pero apenas un 10-20% de la captura se consume a nivel nacional. El resto se exporta a países asiáticos con poco valor agregado. En este sentido, el uso integral de este recurso debe ser considerado, ya que el manto, aletas, cabeza y vísceras se pueden utilizar para diferentes propósitos. En esta revisión, se resumen las principales características de las proteínas del calamar y su aplicación como ingredientes alimentarios, ya sea en la acuicultura o en otras aplicaciones prácticas, así como lo que ha hecho o se está siendo en la industria.

CARACTERÍSTICAS DAS PROTEÍNAS DE LULA E SUA POTENCIAL APLICAÇÃO INDUSTRIAL

Claudia Lizeth Murrieta-Martínez, Víctor Manuel Ocaño-Higuera, Guadalupe Miroslava Suárez-Jiménez e Enrique Márquez Ríos

RESUMO

O aumento global da população tem provocado um aumento pela procura de alimentos, e os produtos do mar não são a exceção. Isto tem levado a uma superexploração das espécies de consumo habitual, pelo qual tem sido apontadas novas espécies para satisfazer as demandas atuais. Uma destas espécies é a lula gigante, cujas características lhe outorgam um grande potencial. Este recurso pesqueiro é um dos mais importantes do México, mas apenas entre 10 e 20% da captura é consumida em nível nacional. O restante é exportado, com pouco valor agregado, para países asiáticos. Neste sentido, o uso integral deste recurso deve ser considerado, já que o manto, aletas, cabeça e vísceras podem ser utilizados para diferentes propósitos. Nesta revisão, são resumidas as principais características das proteínas do calamar e suas aplicações como ingredientes alimentários, seja na aquicultura ou em outras aplicações prácticas, como o que tem sido feito ou se está sendo feito na indústria.