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Short communication

Protein isolates from jumbo squid (*Dosidicus gigas*) by pH-shift processing

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ABSTRACT

There is a growing interest in adding value to the jumbo squid *Dosidicus gigas* fishery. This work describes two extraction procedures for processing muscle to obtain protein isolates with suitable functional properties. The effect on muscle protein solubility and protein recovery of combining freezing and grinding raw materials during storage was evaluated. Processes are based on extraction of protein at acid or alkaline pH and subsequent iso-electric precipitation. About 85% of the initial muscle protein was solubilized at pH 3 and 11. Regardless of the pH used for extraction, about 90% of the protein was obtained after precipitation at pH 5.5. The total yield from both procedures was 75%. Treatments during storage did not significantly affect solubility and yield of protein. Wastewater contained negligible amounts of protein and may be reused. Processing by acid and alkaline extraction are feasible alternatives for obtaining protein isolates either from fresh or frozen squid muscle, which is an important consideration when choosing the most appropriate and inexpensive method to scale up this technology.

1. Introduction

Based on volume of capture rather than upgrading processes, the jumbo squid *Dosidicus gigas* fishery in the Gulf of California is an important economic activity on the Pacific coast of Mexico [1]. Squid muscle is a source of protein that can be incorporated into food products as raw material or protein supplements to manufacture seafood analogues or new products based on its gel-forming properties. According to several research groups [2–5], when minced, squid muscle is used for preparing thermal gels. The presence of proteases affects protein gelation. Besides, acid or bitter taste, related to the presence of some peptides and free amino acids in the muscle [6] and intense ammonia odor produced by high concentrations of non-protein nitrogen products were also reported. Such characteristics discourage direct consumption and further processing. This is one of the reasons why the jumbo squid remains under-utilized.

The conventional surimi washing process seemed to be an alternative for producing functional protein isolates from squid muscle and remove most of the proteases and all the compounds that produce undesirable odor and bitter taste. However, the yield from this process is poor because a large portion of protein is solubilized and washed out.

To overcome these problems, Hultin and his associates developed a pH-aided process for making functional protein isolates from under-utilized muscle protein resources [7,8]. The process is based on extracting muscle protein at pH 2–3 or 11–12, followed by iso-electric precipitation. Precipitated protein is then collected by centrifugation to obtain the protein isolate. In most of those studies, researchers successfully used fresh raw material [7–16]. In our study, the procedure was applied to frozen or aged muscle. This is undertaken because jumbo squid is captured in large volume that cannot be processed immediately during the fishery season; hence, it needs to be frozen and stored for long periods before processing.

In this study, different storage methods were compared to determine the effect on extraction of protein. Additionally, we tested extraction processes under acid and alkaline conditions to produce protein isolates from jumbo squid *D. gigas* to determine yields, organic material present in water used in the process, and protein composition.

2. Materials and methods

2.1. Material and sample preparation

Specimens of jumbo squid *D. gigas* (D'Orbigny, 1835 in 1834–1847) were obtained at the ports of Santa Rosalia (Gulf of California) and San Carlos (Pacific Ocean) in the State of Baja California Sur, Mexico. The mantles were individually wrapped in plastic bags, placed between layers of crushed ice, and transported to the laboratory in <16 h after capture. The skin and connective tissue were removed from the mantle; mantles were chopped into cubes (~1 cm^3) and immediately ground through a 2-mm mesh (Brinkman Instruments, Los Angeles, CA). The squid paste was packaged in plastic bags and stored at 1–2 °C (labeled G) and processed within 10 h or at −30 °C and kept for eight months (labeled GF). A separate squid mantle was frozen before being chopped and ground, packaged, and stored at −30 °C for one year (labeled FGF).

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2.2. Protein solubility curve

To prepare a protein solubility curve, 45 ml aliquots of squid muscle homogenate (muscle:water; 1:10, w:v) were adjusted to a range of pH values (2–12) by adding 2N HCl or 2N NaOH. At each pH level, 1.5 ml homogenate was transferred into 1.7-ml microtubes and centrifuged at 10,000 × g at 4 °C for 20 min. The insoluble pellets were discarded. Soluble protein in the supernatant was assayed in 0.5-ml samples by the Biuret method [17], using bovine serine albumin as the standard. Based on the resulting solubility curves, pH 3.0 and pH 11.0 were selected for further studies.

2.3. Protein recovery via acid and alkaline extraction processing

2.3.1. Protein extraction

Aliquots of 45 ml squid homogenate were adjusted to pH 3.0 or 11.0; homogenates were centrifuged at 10,000 × g, 4 °C for 20 min. The supernatant was separated and the sediment discarded. Protein concentration in the homogenate before centrifugation and in the supernatant after centrifugation was measured using the Biuret method [17]. The soluble protein ratio was calculated by dividing the protein content in the supernatant by the protein content in the homogenate.

2.3.2. Iso-electric precipitation

Supernatants of the acid and alkaline protein extractions were adjusted by adding 2N NaOH or 2N HCl to pH 5.5 at 2 °C to reach the iso-electric point to precipitate proteins in solution. Once pH 5.5 was reached, the mixture was centrifuged at 10,000 × g at 4 °C for 20 min. The supernatants were assayed for protein concentration by the Biuret method. The protein isolate was centrifuged for 20 min at 10,000 × g to remove excess water.

2.4. SDS-PAGE electrophoresis

Samples from the processing, homogenate, extracts, and iso-electric precipitates were analyzed for protein composition and molecular weight using SDS-PAGE, as described by Laemmli [18]. Samples (20 μg) from each treatment were mixed (1:1) with a sample buffer containing dithiothreitol, heated for 5 min in a boiling water bath, then loaded into a 1.5-mm acrylamide gel slab (10% T) assembled in a vertical electrophoresis unit (SE 260, Hoefer, San Francisco, CA). After electrophoresis, the gels were stained for 2 h with a solution containing 0.5% Coomassie Brilliant Blue R-250, 40% methanol, and 7% acetic acid. The excess stain was removed with a solution containing 40% methanol and 7% acetic acid and immediately recorded with an electronic scanner (Umax PowerLook 2100, UMAX Technologies, Fremont, CA).

2.5. Statistical analysis

Results were expressed as means ± S.D. Data were statistically analyzed by one-way ANOVA. The Tukey–Kramer multiple comparison test was used to compare the standard deviation and variance coefficient between data groups and determine significant differences between them at $P < 0.05$ [19], using statistical software (Statgraphics Plus for Windows v. 5.0). Three or four replicates were used in each experiment.

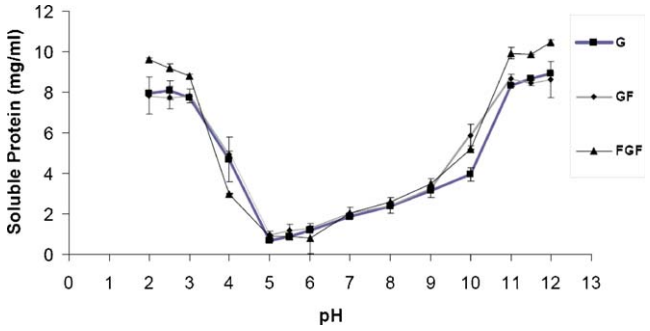

Fig. 1. Effect of pH on solubility of squid muscle proteins. Squid muscle in different preparations was homogenized with 9 volumes of de-ionized water. pH was adjusted using 2N HCl or 2N NaOH. The treatment preparations were G = ground squid muscle, GF = ground and frozen squid muscle, and FGF = frozen–ground–frozen squid muscle.

3. Results and discussion

3.1. Protein solubility and recovery by acid and alkaline processing

Adequate solubility is essential to separate muscle proteins from insoluble material; iso-electric precipitation of neutral solutions will lead to the highest recovery of proteins [14]. Practically, all previous studies using pH-shift solubilizing processing had been used on fish muscle. Our interest was to determinate if the pH-shift method accomplishes this goal for jumbo squid muscle. Solubility of squid muscle proteins, as a function of pH, is shown in Fig. 1. The U-shaped curve, with highest solubility at the crests and lowest solubility in the trough is similar to results from several marine species, such as Pacific whiting [11], rockfish [13], channel catfish [14], and Atlantic croaker [15]. We demonstrated that the process is also suited for squid, as has been previously demonstrated for fish muscle, which are anatomically and biochemically different. The solubility pattern of the protein was independent of the raw material used. Solubility occurs when proteins become positively or negatively charged at acid or alkaline pH, contrary of what happens at the iso-electric point, where protein has no net charge. Solubilization increases by electrostatic repulsion and hydration of charged amino acid

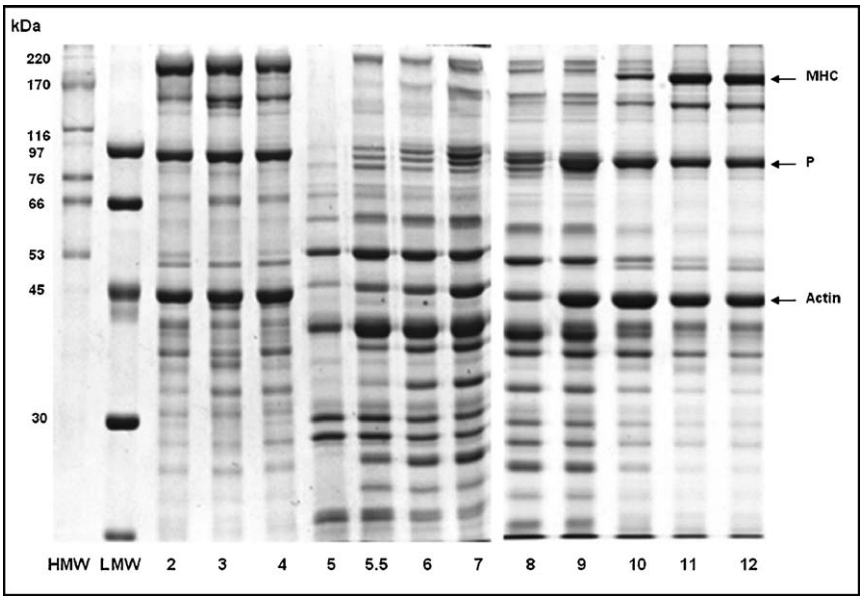

Fig. 2. Effect of pH on solubility of squid muscle proteins, as evaluated by SDS-PAGE. Electrophoresis was performed under reducing conditions. With 20 μg soluble protein loaded at each well. Numbers below each lane indicates pH. MHC = myosin heavy chain; P = paramyosin; HMW = high molecular weight; LMW = low molecular weight.

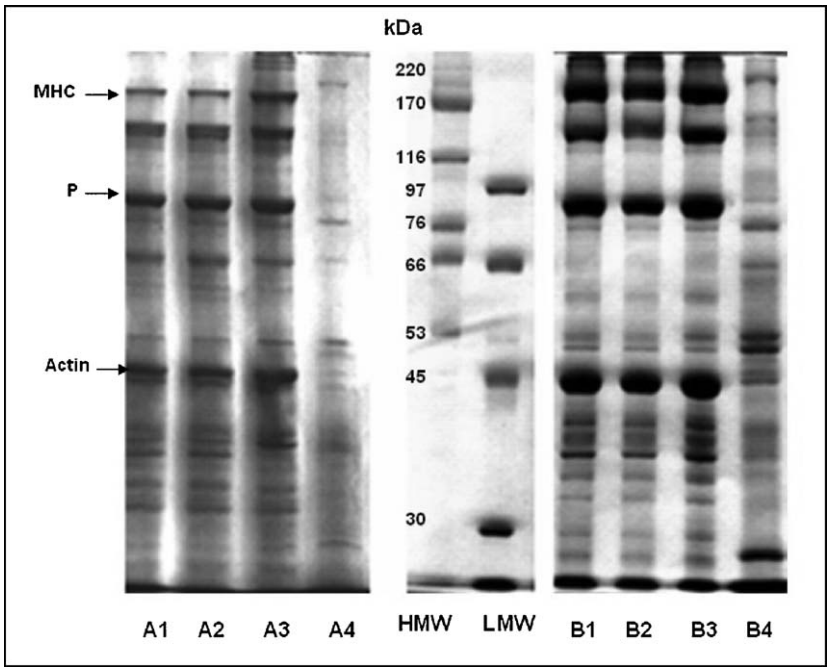

Fig. 3. Protein composition by SDS-PAGE of different samples from acid and alkaline solubilization processes. SDS-PAGE electrophoresis was performed under reducing conditions. 20 μg of each sample was loaded at each well. A = acid process; B = alkaline process; 1 = initial homogenate (pH 3 or 11); 2 = soluble fraction after first centrifugation; 3 = protein isolate; 4 = water at the end of the process; MHC = myosin heavy chain; P = paramyosin; HMW = high molecular weight; LMW = low molecular weight.

residues [20]. The solubility curve was useful for selecting pH 3 and 11 for solubilization (Fig. 2).

Protein recovery was achieved by extraction and precipitation, where ~80% of muscle protein of the initial homogenate was extracted at pH 3 and 11 (Table 1). The increase of net positive charges on squid muscle proteins at low pH probably results from neutralization of negative charges of the carboxylated side chains of aspartic and glutamic acids residues, which have a pK_a ~4.7. The increase in net negative charges at high alkaline pH might result from deprotonation of basic groups, such as the imidizol, guanidyl, and lysyl side chains of histidine, arginine, and lysine and from deprotonation of phenolic side chains, as in tyrosine [9,10].

Yields at acid (84%) and alkaline (85%) pH were not significantly different and are similar to results in herring (*Clupea harengus*) at 92% and 89% [10], rockfish at 80% [13], and mackarel at 80% [21]. de la Fuente-Betancourt et al. [22] reported extraction of muscle proteins from jumbo squid (85%) at pH <3 and >11. In this study, maximum solubility occurred within 5–7 min. Solubilization of muscle protein appears to be almost instantaneous at extreme acid or alkaline pH [23]. The short solubilization time of the squid protein represents an important and attractive advantage for industrial processing.

Another important result is the insignificant differences among diverse ways of storing the raw protein materials, which contradicts the common belief that freezing has negative effects on the solubility of the proteins from raw materials collected for industrial processing [24]. Our results agree with Hultin and Kelleher [7,16] who found that the pH-shift process is successful for extracting muscle proteins from long-frozen fish muscle. Our explanation is that mechanical forces during protein extraction, under moderately extreme acid or alkali conditions, cause complete disruption of the protein structures and yield monomers that allow free interaction with water. At this point, protein conformation would be determined mainly by the pH value. On the other hand, protein from cephalopods had shown high resistance to denaturation from freezing [25,26].

After extraction, soluble protein was precipitated at pH 5.5. The efficiency of precipitation at pH 5.5 was expressed in percent (Table 1, third column). There were no significant differences related to the type of storage. Yields at the iso-electric point were ~90.8%, indicating that most proteins from the previous acid or alkaline soluble fraction were effectively precipitated.

Iso-electric precipitated proteins were collected by centrifugation. Total overall protein recoveries by the acid and alkaline solubilization process were 76.3% and 79.6%, with no significant difference ($P > 0.05$). Hultin et al. [23] reported yields of 85% for pH-shift processes for fish muscle.

Table 1
Protein yields from squid muscle using the acid and alkaline recovery processes.

Process	Solubilized protein	Precipitated protein	Total protein recovery
G			
Acid	85 ± 2.8%	91 ± 5.8%	77.3 ± 6.2%
Alkaline	90 ± 3.0%	90 ± 1%	85.6 ± 5.1%
GF			
Acid	78 ± 2.6%	91 ± 1.7%	71.6 ± 5.0%
Alkaline	79 ± 2.1%	90 ± 1%	72 ± 1.7%
FGF			
Acid	89 ± 2.1%	90 ± 4.7%	80 ± 5.2%
Alkaline	87 ± 3.8%	93 ± 1.3%	81.3 ± 5.0%

G = ground; GF = ground and frozen; FGF = frozen–ground–frozen. No significant differences were observed. Data were statistically analyzed by one-way ANOVA.

3.2. Protein isolates and water recovery

For the acid and alkaline processes, it was possible to recover more than 90% of the water. Iso-electric precipitation yielded 90% of the soluble muscle protein, so the remaining water contained 0.80 ± 0.15 mg ml^{-1} protein. This concentration of protein has low biological oxygen demand and would not cause significant pollution. Reuse of water for new protein recovery processes or multiple reuses

should be evaluated. Efficient water recovering and reuse are additional attractive advantages of the pH-shift process and can easily be implemented at industrial plants where water is an expensive and limited resource, as in Northwestern Mexico; where the jumbo squid fishery is established.

According to Sánchez-Alonso et al. [27], low molecular weight proteins (Fig. 3, columns A4 and B4) and other soluble compounds, such as ammonia and amines, remain soluble in the water and are removed in the supernatant. This results in a protein isolate practically free of unpleasant ammonia odor or taste and a protein isolate useful to prepare seafood analogues without foul odors or taste. Also, it could be used as a protein supplement in many processed food items or in many processed food items in markets where jumbo squid is not appreciated.

3.3. Protein composition and recovery in the acid and alkaline processes

Myosin, paramyosin, and actin are the more abundant proteins in squid muscle [24]. The band intensities of the myosin heavy chain (MHC; ~205 kDa), paramyosin (~108 kDa), and actin (~45 kDa) are high at pH 2–4 and 10–12 and very low at pH 5–6; moreover, at pH 5–6, there is practically no MHC, paramyosin, or actin. These match the solubility curves in Fig. 1. Protein bands of MHC, paramyosin, and actin are present in Fig. 3; they were present in the extraction step at acid or alkaline pH (A1 and B1) and were recovered in the protein isolate (A3 and B3). In the water at the end of the process (A4 and B4), MHC and the other myofibril proteins are not present, having been effectively and efficiently precipitated and collected after the second centrifugation. The soluble proteins remaining in the water are probably the sarcoplasmic fraction of the muscle [23]. SDS-PAGE analysis confirms that recovered protein fractions in the acid and alkali extractions were not different. This is an important consideration when choosing the most appropriate and inexpensive method to scale up this technology.

The evidence indicating that myosin, paramyosin, and actin are present in the isolate at high concentration suggests that protein isolates from squid have excellent functional properties. Unpublished results from our work demonstrated excellent gelation, emulsion, and foaming capacities of the protein isolates from jumbo squid. This promises high expectations in manufacturing a great variety of valuable and acceptable food products.

4. Conclusions

This study demonstrated that most proteins from jumbo squid muscle are readily obtained by extraction at acid and alkaline pH, providing the proteins were efficiently solubilized, even if the raw material was frozen and stored ground, which is in an attractive advantage for processing squid even if frozen for long-time periods. Both alkaline and acid processes provided high protein yields and eliminated bad odor and taste components from the squid muscle.

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