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Effect of thermal process on connective tissue from jumbo squid (*Dosidicus gigas*) mantle

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Abstract

The effect of two thermal treatments (fast freezing at −40 °C and vapor cooking at 100 °C) on connective tissue extract (CTE) from jumbo squid (*Dosidicus gigas*) was investigated. Samples of CTE frozen at −40 °C were taken at 0, 3, 5 and 12 min. Also CTE was cooked at 100 °C and samples were taken at 0, 1, 2.5 and 5 min. Light microscopic observations of CTE after 12 min of freezing showed rupture of fibres. The CTE fibres showed agglutination during cooking time. The CTE insoluble fraction increased with freezing and cooking time. Maximum zeta potential value of untreated CTE was detected at pH 5.0 at +30 mV, meanwhile in the frozen CTE it was detected at pH 7.0 at +30 mV and two peaks (at pH 5.5 and 9.0) were observed at +20 mV in the cooked CTE. One endothermic peak was found at 105.9 °C in the untreated CTE, while in the frozen and cooked CTE the endothermic peaks were found at lower temperatures and enthalpies. Electrophoresis analysis of untreated CTE showed three bands. In the frozen CTE two bands appeared above 200 kDa, and in the cooked CTE, a 45 kDa band disappeared. These results suggest that during freezing and cooking processes there were modifications to molecular bonds that hold the integrity of the structure of the connective tissue of the jumbo squid mantle.
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Keywords: Connective tissue; Jumbo squid; Mantle; Thermal denaturation

1. Introduction

Jumbo squid (*Dosidicus gigas*) is a cephalopod found in abundance off the Pacific coast of Mexico (Martínez-Aguilar, Morales-Bojorquez, Díaz-Uribe, Suárez-Higuera, & Hernández-Herrera, 2004). Although the growth of the fishery in Mexico has been spectacular, great contrasts have characterized this fishery. The local fishing and processing efforts are not supported by appropriate management or technological processes. Therefore, the local fisheries are at a disadvantage when trying to compete for better markets (Luna-Raya, Urciaga-García, Salinas-Zavala, Cisneros-Mata, & Bletrán-Morales, 2006).

Jumbo squid is normally sold in the form of frozen or cooked frozen gutted mantle (Luna-Raya et al., 2006). However, freezing and cooking can affect the functional properties of the proteins resulting in changes on the rheological properties (Ando, Ando, Tsukamasa, Makinodan, & Myoshi, 1999; Badii & Howell, 2002; Paredi, Roldan, & Crupkin, 2006), which alter the texture and eating quality of seafood, thereby causing wastage of a scarce and rich protein source (Paredi et al., 2006). Thus, the proteins and

Abbreviations: CTE, connective tissue extract; SSF, salt-soluble fraction; ASF, acid-soluble fraction; IF, insoluble fraction; DSC, differential scanning calorimetry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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their functional properties are modified in a manner determined by the technological process concerned. The most affected are myofibrillar and stroma proteins (Badii & Howell, 2003; Paredi et al., 2006). Collagen is the major constituent of connective tissues (Foegeding, Lanier, & Hultin, 1996) and has a close relationship with texture.

It is a well-documented fact that during freezing or cooking, proteins undergo conformational changes that lead to their aggregation, thus rendering the muscle harder and tougher, dryer and less succulent (Ando et al., 1999; Badii & Howell, 2002; Paredi et al., 2006). The properties of proteins, collagen in particular, can therefore be used as benchmarks of muscle quality. Even biochemical aspects of frozen squid collagen have received little attention, in some papers on lean fish fillets and squid mantle it is reported that in connective tissue, collagen may also be denatured during frozen storage (Badii & Howell, 2002; Ruiz-Capillas et al., 2002). Whereas, many reports have been published on cooked squid, stating that meat shrinks by about 30% and generally, during the application of heat, collagen in the connective tissue has been considered to affect squid muscle tenderness (Ando et al., 1999, 2001).

The connective tissues are composed of a highly hydrated, amorphous ground substance in which several types of cells, as well as the fibrous proteins collagen and elastin, are embedded (Sikorski & Borderias, 1994). The mechanical properties of the connective tissues depend on the size, orientation, and cross-linking of the collagen fibrils and of elastic fibres, as well as on the proportions of all components of the tissue, including mineral deposits (Bailey & Etherington, 1980). The muscles of squid present a block of muscle fibres made of circumferential bands sandwiched between thinner radial bands that are covered on each side by two connective tissue sheets (Otwell & Giddings, 1980).

Collagen, the major protein of the connective tissues, was detected in a concentration about 3–11.1% in squid mantle (Sikorski & Kolodziejska, 1986). The basic collagen structure, known as tropocollagen, consists of three polypeptide chains, each twisted in a left-handed helix (α-chain), and coiled around each other to form a right-handed triple super helix. Collagen is polymerized through the formation of covalent cross-links. These cross-links become increasingly thermostable as the collagen ages, so that the thermal stability increases. Thermal stability is basically the resistance of the protein molecule to unfolding as a result of any thermal treatment (Bernal, Smajda, Smith, & Stanley, 1987).

On the other hand, there are many publications concerning squid muscle protein solubility (Ando et al., 1999, 2001; Borderias & Montero, 1985; Sato, Ohashi, Ohtsuki, & Kawabata, 1991). These studies are of interest in that many of the functional properties of seafood muscle are related to the solubility of the constituent protein and it is known that the solubility of seafood collagen is generally significantly higher than that of ovine, bovine, or porcine muscle. However this solubility decreases during a frozen storage (Borderias & Montero, 1985; Ruiz-Capillas et al., 2002). Ruiz-Capillas et al. (2002) reported that the collagen solubility of squid mantle decreases after sixteen months at −20 °C. The decrease in collagen solubility during frozen storage could be due to the formation of stable acid and acid keto-imine heat links (Bailey & Etherington, 1980). However, during heat treatment of squid mantle, Ando et al. (2001) and Ando et al. (1999) reported that about 70% of squid mantle collagen was not solubilized even after 30 min of heating in boiling water. Additionally, collagen fibrils in cuttlefish mantle were clearly observed under an electron microscope even after cooking (Ando et al., 1999, 2001). The heat stability of cooked squid muscle is assumed to be related to the presence of heat-stable collagen (Ando et al., 2001).

However, these studies have been done mainly in European or Asian common squids, and no references have been found on the thermal properties of connective tissue proteins from Jumbo squid mantle. On the other hand, thermal collagen stability depends on the hydroxiproline concentration, and this concentration changes between species depending on development temperature and the size of the organism (Sikorski & Borderias, 1994). A better understanding of factors affecting jumbo squid muscle, mainly texture, is important, in developing techniques that will produce improved products and increase its utilization as food.

The present study is an attempt to identify the behaviour of jumbo squid mantle connective tissue proteins subjected to two different thermal processes, freezing and cooking, by considering the changes in some of their structural characteristics: histological observations, solubility, zeta potential, thermal properties through differential scanning calorimetry (DSC), and SDS-polyacrylamide gel electrophoresis. As the thermal activity of individual proteins varies from those present in whole flesh (Wright, Leach, & Wilding, 1977), connective tissue proteins were isolated and analyzed as such.

2. Materials and methods

Jumbo squid organisms used in this study were captured by jigging with a handline in the Gulf of California during autumn season and transported in ice to the Seafood Laboratory at the University of Sonora within 6 h of capture. Five jumbo squid specimens were gutted and mantle was manually skinned.

2.1. Connective tissue extraction (CTE)

The connective tissue proteins were extracted by the method of Montero and Mackie (1992). Chopped squid muscle mantle (100 g) was homogenized with 400 mL 1.4 M NaCl in a biological tissue homogenizer (Biospec Products, Inc., Barthesville, OK), for 1 min. The dispersion was filtered. This procedure was repeated until no muscle fibres were visible to the eye. The resultant connective tissue fibres were stirred into 500 mL 0.05 M NaCl solution

containing 1 mM phenylmethylsulphonyl fluoride (PMSF) for 24 h at 4 °C. After centrifugation at 10,000*g* for 15 min at 4 °C, the supernatant was discarded. Sodium chloride solution (250 mL) was added to 25 g of the precipitate and stirred for 24 h at 4 °C followed by centrifugation at 3000*g* for 20 min at 4 °C. The resultant precipitate was mixed with 250 mL NaCl solution, homogenized for 1 min, stirred 12 h, and finally centrifuged at 255*g* for 30 min. Purified connective tissue was dried between two pieces of filter paper, divided into 1 g portions and immediately were subjected to freezing at −40 °C or cooking at 100 °C processes. Temperature of the middle part of the frozen or cooked connective tissues were measured by a thermocouple (VWR Scientific Inc., 308 West Edgewood, Friedswood, Texas).

2.2. Freezing process

The CTE were frozen at −40 °C with carbon dioxide and acetone solution. Freezing time was established to be 12 min, therefore it was considered as a fast freezing system. During the freezing process, shown as a freezing curve in Fig. 1A, samples were taken at 0, 3, 5, and 12 min. Three frozen CTE portions were sampled and thawed (4 °C) for the analyses.

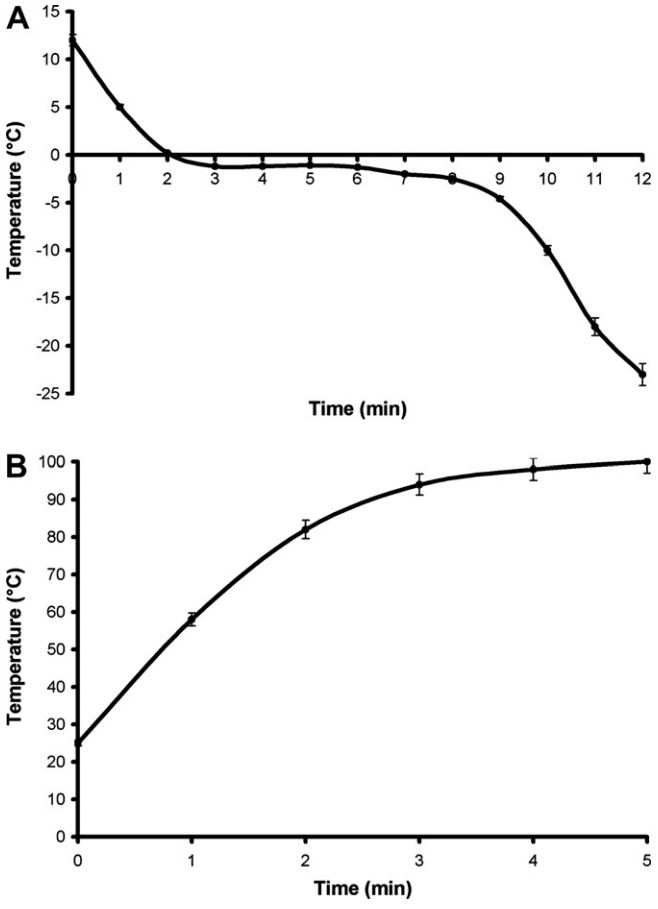

Fig. 1. Freezing (A) and cooking (B) curves of connective tissue extracted from jumbo squid mantle.

2.3. Cooking process

The CTE were cooked by boiling in water. Cooking time was established to be 5 min. Following the cooking curve (Fig. 1B), samples were taken at 0, 1, 2.5, and 5 min. Three cooked CTE portions were used as the samples for the analyses, after cooling for 30 min at room temperature.

2.4. Analyses

2.4.1. Histological observations

Tissue from fresh squid connective tissue and frozen or cooked connective tissue were fixed in 10% formalin. Dehydration process was performed according to Prophet and Mills (1992) by using a tissue processor. After embedding in epoxy resin in the HistoEmbedder, thick sections (5 μm thick) were prepared using a microtome. They were stained with hematohylin and eosin, and observed under an optical microscope.

2.4.2. Salt and acid-soluble connective tissue proteins

CTE proteins were extracted with salt and acid solutions by the method of Borderias and Montero (1985) to obtain soluble and insoluble fractions of CTE proteins at intervals during freezing or cooking process. Purified connective tissue (1 g) was homogenized with 20 mL buffer (0.03 M Tris–HCl pH 7.4) containing 0.5 M NaCl and 1 mM PMSF in a tissue homogenizer for 1 min and stirred for 24 h at 4 °C, followed by centrifugation at 7000*g* for 1 h. This procedure was repeated twice and the supernatants were pooled and called tissue connective proteins salt-soluble fraction (SSF).

The precipitate was mixed with 0.5 M acetic acid containing 1 mM PMSF, 1:20 (w/v), homogenized for 1 min, stirred for 24 h, and centrifuged at 35,000*g* for 1 h. This procedure was repeated once more and the supernatant fractions were pooled and called connective tissue protein acid-soluble fraction (ASF). The precipitate formed the connective tissue protein insoluble fraction (IF). The amount of salt- and acid-soluble connective tissue proteins as well the amount of insoluble connective tissue protein were measured by Bradford method (1976) with bovine serum albumin (Sigma Chemicals, St. Louis, MO) as a standard (1 mg/mL).

2.4.3. Zeta potential determination

Aqueous colloidal dispersions of connective tissue extracted from jumbo squid mantle were prepared as follows for zeta potential determinations. Ten milligrams of CTE were added to 100 mL of an aqueous solution of 1.0 mM NaCl 1 mM, which was used to fix the ionic strength of the solution, and dispersed under magnetic agitation. Then, the pH was adjusted to the desired final value with dilute solutions of nitric acid or sodium hydroxide, and the dispersion stirred during 15 min. The zeta potential (ζ) of CTE was determined at different pH values from electrokinetic experiments. A Zeta Meter 3.0+ unit manufactured by Zeta-Meter, Inc. (New York, NY, USA) was used for this purpose. This apparatus includes

a microprocessor that first measures the electrophoretic mobility of colloidal particles dispersed in aqueous solutions, and then automatically calculates the zeta potential (in mV) using the Smoluchowski equation, which is the most elementary expression for zeta potential calculations from electrophoretic mobility (U) measurements. This equation can be written as: $\zeta = 113,000\frac{\eta}{\varepsilon}U$, where η and ε represent the viscosity and the permittivity of the aqueous medium, respectively. Zeta potentials reported here are the average values obtained by tracking at least 20 different colloidal particles.

2.4.4. Differential scanning calorimetry (DSC)

Thermal behaviour of untreated squid connective tissue and freezing or cooking connective tissues was studied in triplicate by DSC from 20 to 150 °C. The transition temperature and the enthalpy of the proteins were measured using a 1020 Series DSC thermal analysis system (PerkinElmer, Norwalk, CN).The evaluation of the signals was done with a computer using PE Nelson model 1022 from PerkinElmer. The instrument was calibrated for the temperature baseline using indium as a standard. The sample (4–5 mg) was placed in DSC hermetic pans (PE no. 0319-0218) and the test was run at a heating rate of 10 °C/min. An empty capsule was used as a reference (Wright et al., 1977).

2.4.5. Electrophoresis analysis

Only samples of SSF were used for electrophoresis analysis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (1970). The stacking gel and the resolving gel contained 4% and 10% acrylamide, respectively. The sample protein was diluted 1:1 (w/v) in the sample buffer containing 8% SDS, 25% of 0.5 M Tris–HCl (pH 6.8), 20% glycerol, 10% β-mercaptoethanol, 5% deionized water, and 0.03% bromophenol blue. Gels were stained for 4 h in 0.1% Coomassie blue R-250 (Bio-Rad, Richmond, CA) and destined with 50% methanol–10% acetic acid–40% deionized water (50:10:40, v/v/v). The destaining solution was changed at 1-h intervals for 4-h.

2.5. Statistics

For solubility experiments, the mean and standard deviation were calculated and *t*-test was performed to compare the different treatments. The data were analyzed using the JMP4 statistical data analytical software (Statsoft, Tulsa, OK).

3. Results and discussion

3.1. Effect of freezing process on squid connective tissue extracted

3.1.1. Histological observations

Optical microscopy photographs of untreated and frozen squid connective tissue during freezing process were compared in order to establish the effect of freezing on physical damage to the tissue (Fig. 2). The squid CTE cells of untreated tissue showed tight contact with each other and intracellular materials showed intact features (Fig. 2A). At 3 min of freezing, the tissue cells began to detach from each other and some fractures were observed in the tissue fibres (Fig. 2B). At 5 min of freezing, the tissue cells showed severe damage, fractures and large spacing within fibres (Fig. 2C). By the end of the freezing treatment (12 min) evident fibre deformation was observed (Fig. 2D) in the same tissue cells.

The changes detected on squid CTE fibres cells indicated structure changes on CTE proteins, mainly collagen. The disjunction among connective tissue cells that was observed in the present work might be due to disruption of the hydrogen bonds and exposure of residues to the outer surface of the protein molecule, allowing molecules to interact with water as hydrogen-bond donor or acceptor (Badii & Howell, 2003).

3.1.2. Connective tissue proteins solubility

Solubility values for untreated and after 12 min of freezing process CTE proteins (Table 1) indicated changes in the amount of protein soluble in neutral salt and acid solutions (pH 2.55), as a result of treatment. At 12 min freezing process, a significant decrease ($p < 0.05$) of 25.5% and 81.5% were observed in solubility in salt (SSF) and acid fractions (ASF), respectively. In contrast, the formation of insoluble collagen (IF) increased by 182.7% ($p < 0.05$) compared to untreated CTE. Several workers have reported a reduction in protein solubility due to frozen storage, however this results suggests that reduction in protein solubility might be due to frozen storage as well to freezing process and acid-soluble proteins were mainly affected by freezing process, at least under the conditions of the present study. Protein insolubility may lead to changes observed in the rheological properties (Ruiz-Capillas et al., 2002) and toughness of squid detected after only 0.5 months at −20 °C (Ueng & Chow, 1998).

3.1.3. Electrokinetic potential (zeta potential)

Fig. 3 presents the zeta potential of untreated and frozen CTE, which is positive at all pH values investigated between 4.0 and 9.5. The positive zeta potential of CTE might be due to positively charged amino acids within regions of excess of positive charge along collagen fibres (Mertz & Leikin, 2004). The magnitude of the zeta potential, however, depends on the pH and indicates that CTE contains ionizable surface groups that can dissociate. The maximum zeta potential from untreated CTE was +30 mV at pH 5.5 whereas the maximum zeta potential after 12 min freezing was also +30 mV but at pH 6.6. Extrapolation of zeta potential results indicate that the pH where the zeta potential is equal to zero, which gives the isoelectric point of the connective tissue, is at pH 3.5 and 4.0 for untreated and frozen CTE, respectively. Changes in zeta potential values for untreated and after 12 min of freezing process CTE (Fig. 3) may be related to changes in the protein solubility and indicate changes in

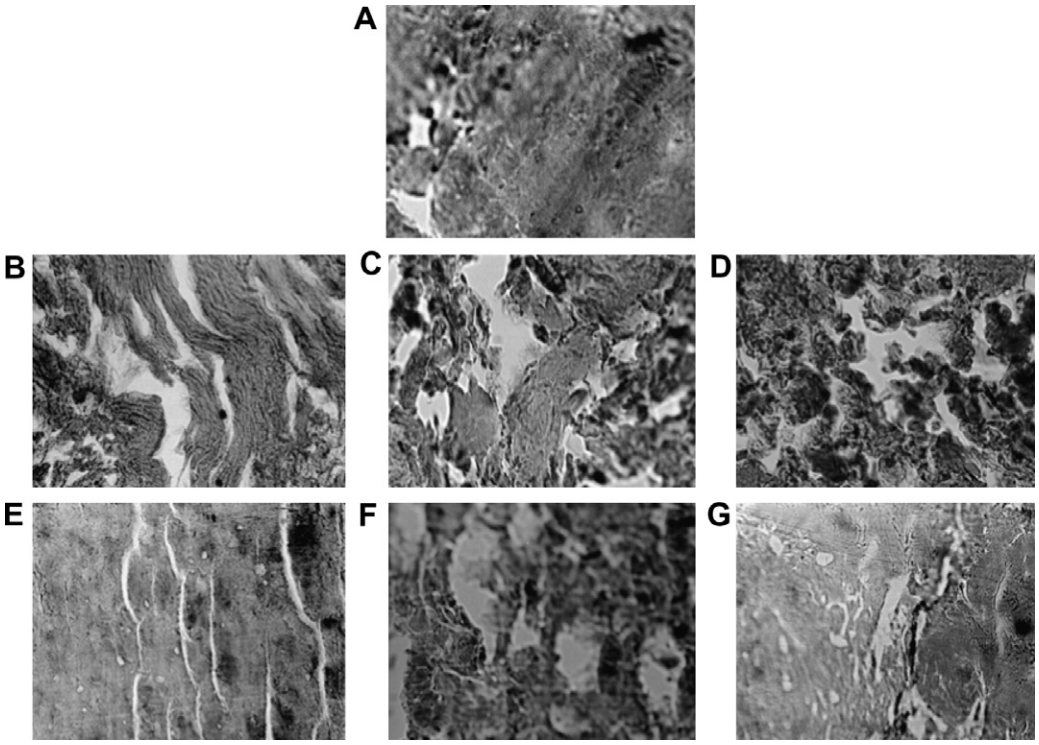

Fig. 2. Histological observations of connective tissue extract (CTE) from jumbo squid mantle during freezing (−40 °C) and cooking (100 °C) processes. (A) Untreated CTE showing tight contact cells; (B) 3 min freezing; (C) 5 min freezing; (D) 12 min freezing; (E) 1 min cooking; (F) 2.5 min cooking; (G) 5 min cooking.

Table 1
Effect of treatment on solubility of connective tissue proteins extracted from jumbo squid mantle

Protein	CTE untreated (%)	CTE freezing 12 min (%)	CTE cooking 5 min (%)
SSF[a]	50.54 ± 1.54	37.66 ± 1.88*	36.7 ± 2.16*
ASF[b]	29.32 ± 1.47	5.43 ± 1.27*	33.7 ± 1.68*
IF[c]	20.12 ± 1.27	56.89 ± 1.84*	29.6 ± 1.48*

Each entry represents an average of three replicates.
* Represents differences with untreated CTE ($p < 0.05$).
[a] Salt-soluble fraction.
[b] Acid-soluble fraction.
[c] Insoluble fraction.

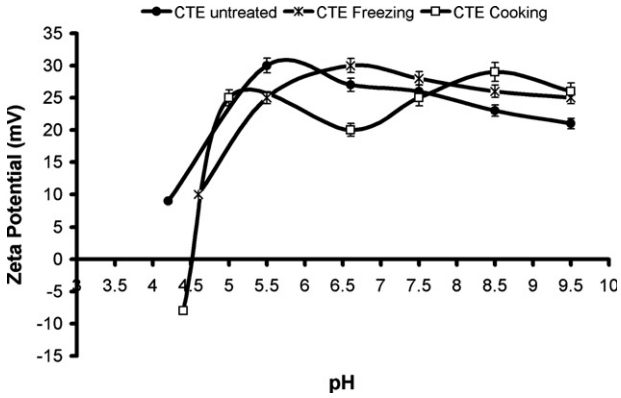

Fig. 3. Electro-kinetic potential (Z potential) of connective tissue extracted (CTE) from jumbo squid mantle. Untreated (●), freezing by 12 min (✱), cooking by 5 min (□).

CTE protein electrochemistry interactions mainly collagen (Badii & Howell, 2003).

3.1.4. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was employed to study the connective tissue in order to evaluate the tissue protein denaturation. The thermograms obtained from untreated CTE were composed of one main peak (Fig. 4) with a transition temperature of 105.92 °C. That sample can be considered to have been in a non-denatured state before scanning since it was not frozen or heat-processed before the assay. Although lower denaturation and shrinkage temperature have been associated with squid collagen (Paredi, Tomas, Crupkin, Añón, 1996) the environmental habitat temperatures of the species has been found to strongly affect collagen thermal stability in seafood (Foegeding et al., 1996). A thermal study of the inner tunic from squid *Loligo pealei* found collagen to denature and melt at 100 °C (Otwell & Hamann, 1979). During freezing process three peaks were observed (Fig. 4) and the relative size of each peak varies with the freezing time, confirm protein denaturation. Over the same time, histological tissue damage was observed as the protein solubility decreased.

3.1.5. Electrophoresis analysis

Electrophoresis analysis revealed different SDS-PAGE patterns for both squid connective tissues, untreated CTE

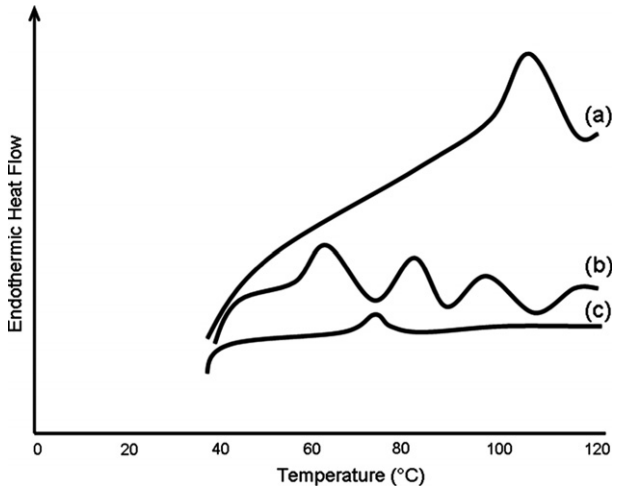

Fig. 4. Differential scanning calorimetry thermograms of connective tissue extracted form jumbo squid (*Dosidicus gigas*). Untreated CTE (a), CTE freezing for 12 min (b), CTE cooking for 5 min (c).

and during the freezing process (Fig. 5 gel A). As shown in Fig. 5 (lane b), the electrophoresis pattern from untreated CTE showed three bands with molecular weights of approximately 200, 97 and 45 kDa. It was reported that the muscle "stroma" consists of some proteins, including, collagen, elastin, and reticulin (Sikorski, Kolakowska, & Pan, 1990). Therefore, some of the bands observed could correspond to collagen, such as that found in abalone as 116 and 205 kDa bands (Yoneda et al., 1999); to elastin proteins, as reported for salmon with a molecular weight of 45 kDa (Chow, Boyd, Iruela-Arispe, Wrenn, Mecham, & Sage, 1989). There is also the possibility of presence of paramyosin, a myofibrillar protein, which has been reported as a 97 kDa band (Sotelo, Piñeiro, Pérez-Martín, & Gallardo, 2000).

After 3 min of freezing process, one band over 200 kDa (Fig. 5 gel A, lane c) was observed and at the end of the treatment. A new band between 116 and 200 kDa (Fig. 5 gel A, lane e) also was detected, indicating protein alterations. The bands located above 200 kDa (Fig. 5 gel A, lane e) may also correspond to adducts of fragments produced by the breakdown of proteins, as observed by different researchers during degradation of squid mantle (Hernández-Andres, Gómez-Guillen, Montero, & Pérez-Mateos, 2005). These alterations confirm more damage to the tissue, as observed by optical microscopy.

3.2. Effect of cooking process on squid connective tissue extract (CTE)

3.2.1. Histological observations

Light microscopic photographs were compared on squid untreated CTE and cooked CTE at different times (Fig. 2). After 1 min, a gross distortion of tissue was observed (Fig. 2E). After 2.5 min fibres of connective tissue showed signs of "melting" and "gelatinization" (Fig. 2F). At the end of cooking time (5 min) fibres

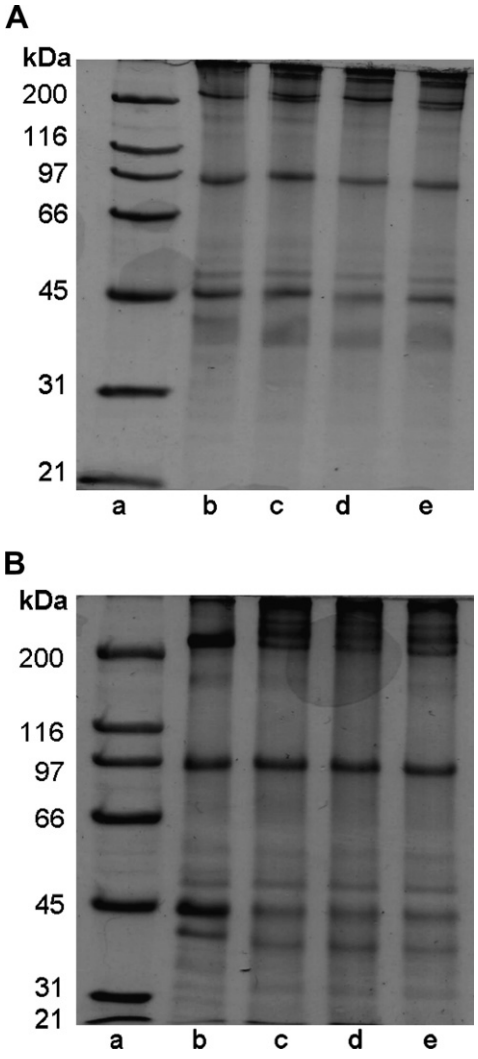

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis from jumbo squid mantle connective tissue extracted proteins. Gel A: Molecular weight standards (lane a); untreated CTE (lane b); 3 min freezing (lane c); 5 min freezing (lane d); 12 min freezing (lane e). Gel B: Molecular weight standards (lane a); CTE untreated (lane b); 1 min cooking (lane c); 2.5 min cooking (lane d); 5 min cooking (lane e).

observed in the untreated CTE could not be found and there was a complete absence of structure (Fig. 2G). Heat coagulation of proteins and gelatinization of connective tissue were obvious, as observed elsewhere (Otwell & Giddings, 1980).

3.2.2. Connective tissue proteins solubility

The solubility in salt solution (SSF); in dilute acetic acid (ASF) and insoluble CTE (IF) were used as indices of structural protein modification after heating treatment. Table 1 shows how much squid CTE remained soluble in salt or acid after heating processes. Meanwhile SSF decreased, the ASF and the IF showed a significant increase ($p < 0.05$) after processes. The modification in proteins solubility confirmed protein denaturation. The proteins were converted to gelatin, as was observed in histological study.

3.2.3. Electrokinetic potential (zeta potential)

When collagen is heated to temperature greater than 60 °C, an irreversible shrinkage takes place. This shrinkage is caused by the breakdown of the various cross chain linkages between the polypeptides chains causing a change in the isoelectric point. Thus, heated CTE was expected to show different zeta potential results than untreated CTE. After heating processes two peaks (Fig. 3), one at pH 5.18 with a zeta potential value of +20 mV and the second at pH 8.77 with a zeta potential value of +25 mV, were observed. Moreover, the zeta potential reverses sign from negative to positive at pH 4.5. During heating the structure cohesion forces of collagen are reformed to other linkages and such changes induce that basic group strength is increased while acid group is decreased.

3.2.4. Differential scanning calorimetry (DSC)

While microscopy was used to observe structural changes of heated CTE fibres, thermal denaturation of CTE proteins was obtained by DSC (Fig. 4). During heating, structural CTE proteins decrease in dimension upon reaching their thermal denaturation temperatures and cause shrinkage of the ETC fibrils and tissue. It is assumed that thermal denaturation of CTE started at first min of heating, because very small peaks at lower temperature occur in thermograms (Fig. 4).

3.2.5. Electrophoresis analysis

Fig. 5 gel B presents the electrophoretic pattern of the CTE proteins fraction from squid CTE before being heating treatment at 100 °C and after 1, 2.5 and 5 min. In descending order of molecular weight, band about 97 kDa was recorded in all cases. As in the case of freezing processes of the CTE, a weaker band was observed at 200 kDa (Fig. 5 gel B, lane e). Besides that, weaker bands were detected between 45 and 31 kDa (Fig. 5 gel B, lane e). As in freezing processes, a band above 200 kDa (Fig. 5 gel B, lane e), was also detected. These results indicate a higher denaturation in squid CTE proteins at 5 min of heating processes.

4. Conclusions

Under the conditions of this work, it was evident that the freezing and cooking processes induced fracture and detachment of squid connective tissue cells. The behaviour of connective tissue of jumbo squid mantle during one freezing or cooking processes has not been reported previously; therefore our results might be a first step in the understanding of the alterations occurring during the processing of the jumbo squid mantle.

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