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Method for producing a functional protein concentrate from giant squid (*Dosidicus gigas*) muscle

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Abstract

The conventional way of processing a protein concentrate (surimi) (and further gelation) from squid muscle poses a number of difficulties, essentially because of the peculiarities of the myofibrillar proteins, which cause extensive very high autolysis and, sometimes, off-flavours and bad taste in the muscle. To overcome these problems, a new procedure has been devised for processing this functional protein concentrate from giant squid (*Dosidicus gigas*) muscle. It is based on the solubilization of the mantle at very low ionic strength and neutral pH (0.16 M NaCl and 0.1% $NaHCO_3$) with 250 ppm of EDTA and further acid precipitation (pH 4.7–4.9) of much of the muscle protein, eliminating the substances responsible for bad taste and odours in the discarded supernatant. Subsequent thermal gelation should be achievable in only one stage, at 90 °C, after adding 0.2% $Ca(OH)_2$ and 1% NaCl. With this processing, gels of about 400 g cm of gel strength can be obtained.

Keywords: Surimi; Protein concentrate; Squid; Gelation

1. Introduction

Cephalopod muscle has the potential to be used for manufacture of protein concentrate (surimi) to prepare seafood analogues and other new products, based on the gel formation of muscular proteins, given that the muscle is white, has little flavour and virtually no fat and is in abundant supply throughout the world.

Published studies on the gelation of squid muscle highlight a number of difficulties regarding the peculiarities of the myofibrillar proteins and very extensive autolysis in this muscle. The peculiarities of proteins in molluscs include a different major myofibrillar protein, paramyosin, and the myofibrillar proteins solubilize at very low ionic strength (Tsuchiya, Yamada, & Matsumoto, 1978).

The direct use of minced squid muscle in the preparation of thermal gel products (Gomez-Guillén, Borderías, & Montero, 1997; Gomez-Guillén, Montero, Solas, & Borderías, 1998; Gomez-Guillén, Hurtado, & Montero, 2002; Lee, 1984) and freeze-texturized products (Maza & Rivas Plata, 1990, 1994) poses technological problems, one of the most important of which is the presence of proteases that interfere with gelation (Ayensa, An, Gomez-Guillén, Montero, & Borderías, 1999; Ayensa, Montero, Borderías, & Hurtado, 2002; Konno & Fukazawa, 1993). These enzymes are mainly metalloproteases which cleave the myosin molecule selectively into heavy meromyosin (HMM) and light meromyosin (LMM) (Konno & Fukazawa, 1993).

Konno and Fukazawa (1993) reported that the mantle muscle of common squid (*Todarodes pacificus*) contains at least two types of proteolytic enzyme: one cleaves myosin into heavy meromyosin and light meromyosin, and the other cleaves myosin into S-1 and rod subfragments. In a different kind of squid (*Dosidicus gigas*), Konno, Youngie, Yoshioka, Shinho, and Seki (2003) determined that the proteolytic enzymes involved in autolysis were metalloproteases and, when the medium contained 2 mM EDTA, this autolysis was significantly suppressed. The same

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authors further reported that the concentration of NaCl in the medium influenced the protease capacity, which presented a maximal rate at approximately 0.3 M NaCl.

Another problem with some squid species, specially *Dosidicus gigas*, is the intense odour of ammonia given off by its muscle, owing to the presence of high concentrations of non-protein nitrogen (NPN), such as nitrogenated volatile bases (NVB) and trimethylamine (TMA) (Maza, Salas, Rosales, & Castro, 2003; Sánchez-Brambila, Alvarez-Manilla, Soto-Cordova, Lyon, & Pacheco-Aguilar, 2004). This species also presents a noticeable bitter taste, associated with certain peptides and free amino acids (Sánchez-Brambila et al., 2004). For this reason, although there are methods for partially eliminating such compounds in whole muscle by washing (Maza et al., 2003), more thorough washing would be needed to totally eliminate the unpleasant odours and partially eliminate the proteases. This species, found in abundance off the West Coast of Mexico and Peru, is not particularly liked because of these problems.

Preparation of a concentrate, without a large number of enzymes and low molecular weight substances that give off bad odours, should be easy if the muscle could be washed, as in the preparation of fish surimi. However, because of the easy solubility of muscular proteins in squid muscle, this cannot be done. To overcome this problem, a new procedure has been devised to produce a functional concentrate from cephalopod muscle (Careche, Borderias, & Sánchez-Alonso, 2004) to be used alone or mixed with other surimis to prepare restructured products based on protein gelation, such as seafood analogues. The objective of the present work was to design a procedure based on isoelectric protein precipitation of muscular proteins for preparing a tasteless protein concentrate (surimi), with good gel-forming capacity, from *Dosidicus gigas* muscle. The procedure is easy to scale up to an industrial level.

2. Materials and methods

2.1. General

In order to obtain a protein concentrate from *Dosidicus gigas* muscle, as odourless and tasteless as possible and with the greatest gel-forming capacity, a procedure was followed whose stages include solubilization, filtering, isoelectric precipitation, neutralization of the concentrate, addition of cryoprotectants and freezing (Careche et al., 2004).

The raw material is frozen mantle, 3 cm wide and about 1 m long. *Dosidicus gigas* was caught in the Gulf of California using the hand-jig method. Following capture, the specimens were gutted and the mantle was separated from the tentacles and frozen on board to −20 °C in a plate freezer. The mantles were shipped frozen at −20 °C to the laboratory in Madrid. The time-lapse between capture and arriving at the laboratory was approximately 2 months.

Before starting the processing, the inner and outer fasciae, which are composed of connective tissue that is unattractive and would not be wanted, were removed. In the laboratory these fascias are removed manually when the mantle is partially thawed; in a pilot plant they can be removed by passing the tempered mantle through the 3-mm holes in the drum of a Baader 694 fish de-boning machine (Baader, Lübeck, Germany).

2.2. Reagents

The brand of the reagents HCl, NaOH, $NaHCO_3$ $CaCl_2$ and $Ca(OH)_2$ was Panreac Química S.A. (Montplet and Estaben S.A., Montcada i Reixac, Barcelona, Spain).

2.3. Ingredients

Food-standard sodium chloride (NaCl), saccharose, sorbitol and sodium tripolyphosphate from Panreac Química S.A. were used. The starch was Clearam Ch 20 from Roquette Freres, supplied by Levantina Agrícola Industrial S.A. (Laisa), Barcelona, Spain. Bovine plasma protein (BP) extract (AMP 600N) was obtained from American Meat Protein Corp., Inc. EDTA was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Antifoamants

The following antifoamants were used in the study: antifoamant from CIBA Especialidades Químicas, Barcelona, Spain; DOW 1510 silicon antifoaming agent from DOW Corning Europe, Brussels, Belgium; and antifoaming agent E-471 from Degussa Texturant Systems España, S.A, Rubí, Barcelona, Spain.

2.5. Solubilization of muscle protein

After processing in the de-boning machine, the minced muscle was placed in a refrigerated homogenizer (Stephan UM5, Stephan u Söhne GmbH & Co., Hameln, Germany) with NaCl solutions ranging from 0 to 0.8 and 0.1 M sodium bicarbonate in some cases. The muscle: solvent proportion was 1:5. Solubilization time was 5 min.

2.6. Protein precipitation at the isoelectric point

The pH of the solution of muscle was modified up to 4.5–5; the solution was left for 30 min to allow proper precipitation of the myofibrillar protein (based on prior experience). The sample was then centrifuged to precipitate the protein and separate it from the solvent, using a BECKMAN model J2-MC centrifuge with a JA-14 rotor revolving at 2500*g* at 2 °C during 5 min. A vertical centrifuge (model OKA-2, Westfallia Separators Ibérica S.A., Barcelona, Spain) and a decanter (model NX-409, Alfa Laval FME A/S Tumba, Sweden) were also used.

2.7. Addition of cryoprotectants and freezing

The protein concentrate was mixed in a Stephan UM5 cutter with 4% sorbitol, 4% saccharose and 0.5% sodium tripolyphosphate, cryprotectants habitually used in fish surimi. The mixing time was 2 min at slow speed. The process was carried out under vacuum conditions (−0.9 bar) at a temperature of 2 ± 2 °C. The concentrate was then frozen in an air freezer until the thermal core reached −20 °C.

2.8. Gelation

Samples of 600 g of frozen protein concentrate were tempered and placed in the Stephan UM 5 cutter, which had first been chilled to 2 ± 2 °C. The sample was neutralized with NaOH and $Ca(OH)_2$, separately, and homogenized for 2 min, adding cold water as necessary to obtain a final gel moisture of 75%. Then, 1% of NaCl was added and the sample homogenized for a further 5 min at slow speed, vacuum (−0.9) and 2 ± 2 °C. The temperature increased due to generated by the blade friction but was less than +8 °C at all times. After that, the sample was stuffed into a 35 mm diameter Krehalon skin (Amcor Flexibles Hispania, S.A., Ganollers, Barcelona, Spain), using a manual extruder, and heated for 30 min in a water bath at 90 °C. It was then placed in ice-water for 1 h and stored in a refrigerator for 24 h before the package was opened and the appropriate analyses were run.

2.9. Analyses

2.9.1. Proximate analyses

Moisture, fat and ash content of the raw samples were determined (AOAC, 1995) in quadruplicate. Crude protein content was measured in quadruplicate using a Nitrogen Determinator LECO FP-2000 (Leco Corporation, St. Joseph, MI).

2.9.2. Determination of pH

The pH was determined, in triplicate, using a pH meter (Radiometer PHM 93, Copenhagen, Denmark) on a homogenate of 10 g sample in 100 ml distilled water.

2.9.3. Determination of foam inhibition

The antifoaming capacity of the agents used and their capacity to stabilize the foam over time were verified. The agent was used in the dosages recommended by the makers (1 and 2 ml). The antifoaming agent was tested in two stages of the process: in solubilization and in isoelectric precipitation with HCl. In both instances the antifoaming agent was added to the muscle/solvent mixture, which was then stirred mechanically for 2 min in an Omni-Mixer (Model 17196, OMNI International, Waterbury, USA). Immediately after that, the liquid was poured into a 50 ml test tube and the volume of the foam was measured after 0, 30, 60 and 90 min at 5–10 °C.

2.9.4. Measurement of colour

Colour measurements consisted of determining L^*, a^* and b^* using the CIELab scale (Park, 1995; Young & Whittle, 1985) where L^* is the parameter that measures lightness $+b^*$ the tendency towards yellow and $+a^*$ the tendency towards red. Measurements were obtained in a HunterLab model D25-9 colorimeter (D45/2°) (Hunter Associates Laboratory Inc., Reston, VA, USA), with measurements standardized with respect to the white calibration plate. Whiteness was determined using the following formula: $100 - [(100 - L^*)^2 + a^{*2}b^{*2}]^{1/2}$ (Park, 1994).

2.9.5. Electrophoresis (SDS–PAGE)

Protein samples were treated with a denatured solution consisting of 5% 2-β-mercaptoethanol, 2.5% sodium dodecyl sulphate (SDS), 10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.002% bromophenol blue, following Hames (1985), and the final average concentration was adjusted to 2 mg/ml. The sample was heated for 5 min at 100 °C. Electrophoresis assays were performed on a PhastSystem apparatus (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) using 12.5% polyacrylamide gels supplied by Pharmacia. Electrophoretic conditions were 10 mA, 250 V and 3.0 W, temperature 15 °C. Protein bands were stained with Coomassie brilliant blue, commercialized by Pharmacia as "PhastGel Blue R" tablets. An aqueous solution of 30% methanol and 10% acetic acid was used for de-staining, and a solution of 5% glycerol and 10% acetic acid as a preservative. The reference standard used for molecular weights was a commercial high molecular weight (HMW) calibration kit from Pharmacia, consisting of: myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa). The disappearing rate of the myosin heavy chain band on the electrophoresis profile was analyzed by the programme ID-Manager v. 2.0 (TDI S.A, Madrid, Spain) on a computer with a scanner (hp scanjet 4470c).

2.9.6. Analysis of gel strength

The analysis was conducted using a TA-XT2 Texture Analyser (Texture Technologies Corp., Scarsdale, NY). The gels, with the skins removed, were cut into cylindrical pieces 3.5 cm wide and 3 cm thick and were tempered at 20 °C prior to measuring. A penetration test was performed, in which the gel was penetrated to breaking point. Breaking force (N), breaking deformation (mm) and gel strength (work to penetration) (g cm) were determined in the force–deformation curves. All the determinations were carried out at least in quadruplicate. The samples (gels) were penetrated to breaking point using a spherical-ended stainless steel plunger (diameter = 5 mm) attached to a 50 N cell connected to the cross-head of a texturometer. The cross-head speed was 0.2 mm/s.

2.9.7. Gelation profile

Dynamic viscoelastic studies were performed on a Bohlin CSR-10 rheometer rotary viscometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone and plate geometry (cone angle 4°, gap = 1.50 mm). A sample of protein concentrate with cryoprotectants was thawed, then mixed with 3% starch and 1% NaCl. It was then heated from 5 to 80 °C at a scan rate of 1 °C/min, frequency 0.5 Hz, and target strain 0.02 mm. The elastic modulus (G'; Pa) was plotted as functions of temperature. The results were the averages of at least two determinations.

2.9.8. Sensory analysis

Sensory analysis was performed by a panel of five semi-trained tasters chosen from laboratory staff using non-structured scales for measuring the intensity of three characteristics: ammonia odour, acid taste and bitter taste. The anchor words placed at the ends of a 12 cm line were ''very much'' (12 cm) and ''nothing'' (0 cm). The judges met in five preliminary sessions to taste different sizes of boiled muscle squid with different degrees of ammonia odour. The samples analyzed in triplicate were: (a) odour and taste of boiled mantle strips of raw material; (b) odour of protein concentrate after the isoelectric precipitation process; (c) gels after processing, using the different additives for gelation described in this paper (EDTA, NaOH, $Ca(OH)_2$).

2.9.9. Statistical analysis

One-factor ANOVA was performed to see if there were significant differences between the treatments used. The method used for distinguishing between the means of the groups was Fisher's least significant difference (LSD) procedure with a confidence level of 95%; a post hoc Student-Newman–Keulis test was also performed, with a significance level of $p < 0.05$.

3. Results and discussion

3.1. Proximate analysis

The major constituents of the raw squid muscle used were: moisture 84.3 ± 0.3%, crude protein 14.5 ± 0.2%, crude fat 0.6 ± 0.1% and ash 0.6 ± 0.05%. The pH varied among individuals, averaging 6.4 ± 2.

3.2. Solubilization

Minced, partially refined muscle was solubilized with an aqueous solution of NaCl. Several solute/solvent ratios were tried in order to make the solution viscous enough to be easily manageable during processing and to be able to centrifuge it afterwards. It was found that the minimum ratio for satisfactory processing was 1:5 (muscle:solution).

In order to ascertain the minimum NaCl concentration required, the muscle solubility was determined with several NaCl concentrations in the solvent liquid (Fig. 1); it was found that, with NaCl molarity of 0.16 and upwards,

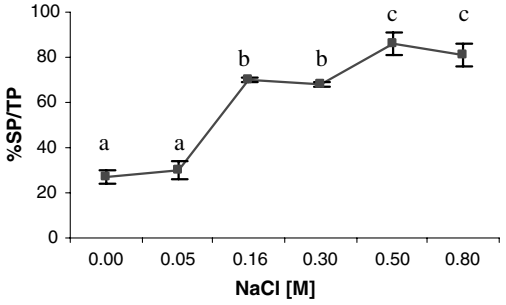

Fig. 1. Percentage of muscular protein solubility expressed as soluble protein (SP) in relation to total protein (TP), depending on different NaCl concentrations in the solvent Different letters indicate significant differences ($p < 0.05$) between samples at different concentrations.

70% or more of the muscle protein was solubilized. Given that protease activity was maximum at 0.3–0.5 M NaCl (Konno et al., 2003) and that the solutions could not contain more than 0.5 M NaCl, since the final concentrate had a strong salty taste, it was decided to use a solution with 0.16 M NaCl to minimize protease activity, even although this meant some loss of protein. To achieve good solubilization, it is important to adjust the pH of the solution to between 6.5 and 7; this can be achieved, either by adjusting the solution's pH with 1 M NaOH or by adding enough 0.1% sodium bicarbonate to the solution to make a buffer of around pH 6.7.

It is important to produce as little foam as possible in homogenizing, since the foam will interfere in the subsequent decanting and centrifuging stages. A food-grade silicon antifoaming agent is therefore recommended.

Regarding the use of antifoaming agents, a study of three different commercial agents showed that both DOW 1510 and E-471 silicones are effective in this process.

3.3. Filtering of solution

In order to refine the myofibrillar protein solution and eliminate fasciae, the solution is passed through nylon fabric with 300-μm pores in a laboratory. In a pilot plant, these residues are readily removed with a decanter or a screw filter with an orifice of 300–400 μm.

3.4. Isoelectric precipitation of protein

Protein precipitation was studied at several different pH values by adding 0.55 M HC1 to the muscle solution and a pH between 4.5 and 5 was enough to precipitate about 90% of the protein (Fig. 2). The precipitate was collected using a laboratory centrifuge and the yield attained with this procedure was 72 ± 2% (in dry matter). In a pilot plant, a vertical continuous centrifuge can be used, or a decanter revolving at around 5000*g*. With the vertical centrifuge used, the yield was 54 ± 2%, and with the decanter used it was 50 ± 2%. Low molecular weight proteins, many of which are probably proteases, are removed with the supernatant liquid

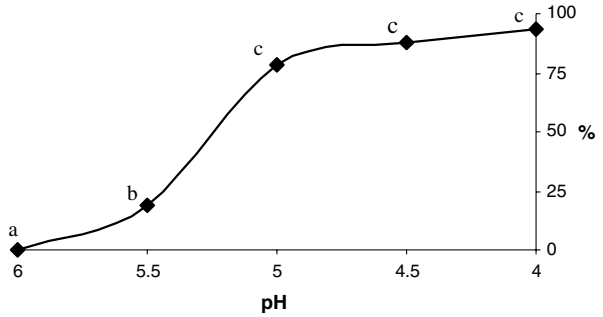

Fig. 2. Protein precipitation (%) from muscle solutions in relation to total protein at different pH values of the solution. Different letters indicate differences between samples.

(Fig. 3) and other soluble compounds such as ammonia and amines (Maza et al., 2003) which give this muscle an unpleasant taste, especially when the raw material is from large specimens.

3.5. Frozen storage

After the cryoprotectants had been added, the resulting product was free of impurities, and had a whiteness index of 80 and a lightness (L^*) index of 79.5.

In order to investigate the effect of pH level in the concentrate during frozen storage, one part was neutralized with 1 M NaOH and another was stored frozen with the acidic pH it already had (≈5). Fig. 4 shows that there was a slow but steady loss of gel strength over time in the case of the sample stored frozen in an acidic medium. It is interesting to note that this loss was due to reduced breaking force and not to reduced breaking deformation. However, when the concentrate had been neutralized before freezing and was kept in frozen storage, the gel strength was virtually zero one day after freezing. In light of these results, further study is required on the cryostabilization of this protein concentrate.

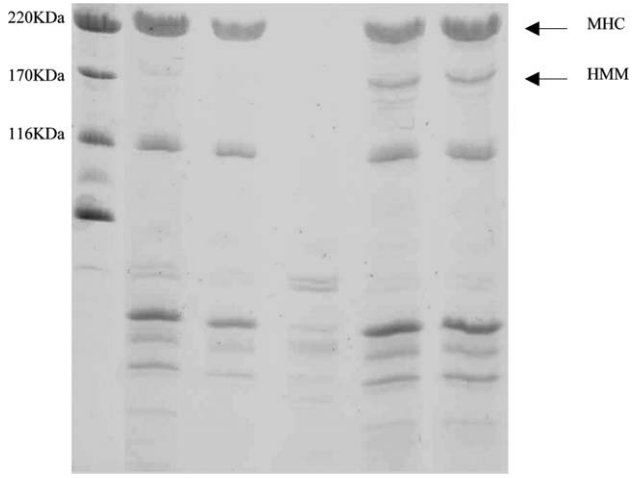

Fig. 3. Electrophoretic profile of materials at different stages of the protein concentrate process: (1) HMW marker; (2) muscle; (3) solubilized muscle; (4) supernatant; (5) precipitated protein concentrate; (6) neutralized protein concentrate.

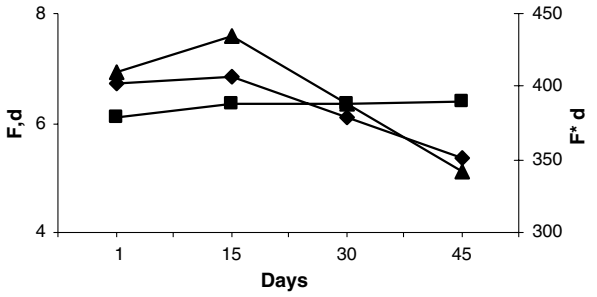

Fig. 4. Force to rupture (◆, F) in N, deformation to rupture (■, d) in mm and gel strength of gels (▲, $F \times d$) in g cm prepared with the frozen protein concentrate during 45 days of storage.

3.6. Protein degradation by proteases

Myofibrillar protein degradation was studied by electrophoresis over 24 h in a stored chilled muscle extract dissolved in 0.5 M NaCl at 2–5 °C. As Fig. 5 shows, the myosin heavy chain (MHC) decreased gradually, disappearing by the end of 24 h at 2–5 °C. At 25 °C, the MHC disappeared at a level of 93% after 2 h and completely after 4 h of storage. The decrease in the MHC coincided with increases in the other HMM bands at 132 kDa and LMM bands at 100 kDa. This degradation is due to the fact that squid muscle presents high protease activity (Ayensa et al., 1999; Konno & Fukazawa, 1993; Konno et al., 2003), so it is important to do the processing as soon as possible and at a lower temperature.

Fig. 3 shows the electrophoretic pattern of the proteins during the different stages of protein concentrate processing (1 h at 3–8 °C). The degradation bands do not appear merely as a consequence of solubilization; however, after precipitation there is a thickening of the HMM band produced by the degradation of the MHC. The MHC band

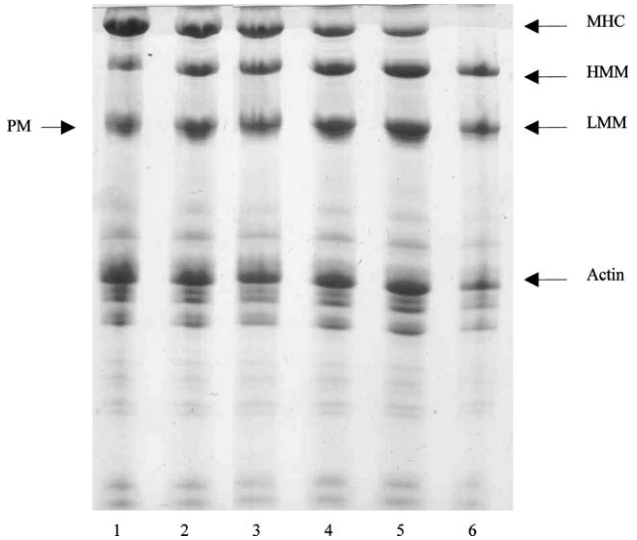

Fig. 5. Changes, over time, in the electrophoretic profile of muscular proteins in a chilled muscle solution stored in 0.5 M NaCl: (1) 0 min; (2) 30 min; (3) 2 h; (4) 4 h; (5) 6 h; (6) 24 h. MHC, myosin heavy chain; HMM, heavy meromyosin; LMM, light meromyosin: PM, paramyosin.

accounts for 49.8% of all the bands found in the solubilized protein, while, in the precipitate, the MHC accounts for 35.2%. This lower concentration is due to proteolysis, as a result of which the HMM band (132 kDa), due to degradation of the MHC, appears in the profile for the precipitate. In the electrophoretic profile of the supernatant, low molecular weight proteins are observed, and many of them are enzymes that must be removed in the processing.

Table 1 shows the rate of disappearance of the MHC, depending on the presence or absence of EDTA in the muscle solution, at two different temperatures. With 250 ppm EDTA, at 2–5 °C, the enzymic action was inhibited for 1 h, while, without EDTA, the rate of degradation of the myosin heavy chain was 18.4%. The EDTA also inhibited enzymic action when the temperature was raised to 25 °C. These data are consistent with the findings of Konno et al. (2003).

3.7. Gelation characteristics

The protein precipitate contained approximately 80% moisture and around 1% NaCl and the pH was 4.5. For gelation, the concentrate was neutralized in two ways: first, with a solution of 1 M NaOH (higher concentrations cause spontaneous gelation when the NaOH comes into contact with the concentrate), and second with powdered 0.2% calcium hydroxide ($Ca(OH)_2$), in both instances until the pH of the batter was close to 7, mixed in the Stephan UM5 cutter. Neutralization is easily achieved in both instances, but it is better to use powdered $Ca(OH)_2$ since no water needs be added to the sample. Neutralization was also attempted with sodium bicarbonate, but this was found to be unsuitable because of bubbling on further heating. The resultant gel strengths, after being neutralized with either sodium hydroxide or calcium hydroxide, were 408 ± 15 g cm.

The gelation profile (G', elastic modulus) is shown in Fig. 6. G' values of this concentrate are 10 times higher than in a homogenate prepared from squid muscle (Gomez-Guillén et al., 2002). Moreover, the typical gelation profile of squid muscle (Gomez-Guillén et al., 1997; Gomez-Guillén et al., 2002), in which the plot of elastic modulus (G') exhibits a considerable depression in a range of 30–50 °C, does not occur in the case of this concentrate, probably because a considerable part of the enzymic fraction has been eliminated. Subsequently, from 45 °C upwards there is a linear increase in G' up to 80 °C, as a consequence of progressive thermal aggregation of myofibrillar proteins in the development of the gel structure.

Table 1
Disappearance (%) of MHC in a muscle solution in 0.5 M NaCl during different storage times and temperatures with or without 250 ppm EDTA

	Time (h)	0 ppm EDTA	250 ppm EDTA
$T = 2$ °C	1	18.4	0.0
	2	39.3	22.7
$T = 25$ °C	1	72.0	2.6
	2	100	33.5

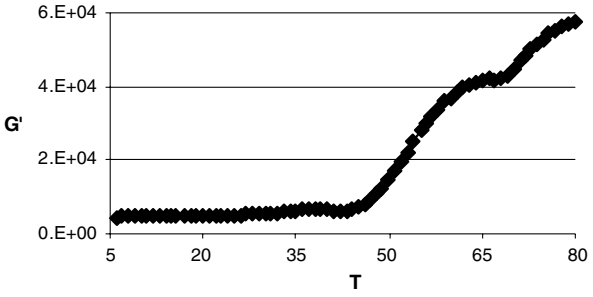

Fig. 6. Changes in elastic modulus (G') during thermal gelation at 1 °C/min of protein concentrate homogenized with 1% NaCl.

Unlike the muscle of other squids, the profile gives no indication that this is a protein susceptible to setting, which would be apparent as a small peak in the 40–50 °C range (Sano, Noguchi, Tsuchilla, & Matsumoto, 1989).

3.8. Sensory characteristics

Samples used as raw material scored: ammonia odour, 6 ± 0.4; acid taste, 7 ± 0.8; bitter taste, 5 ± 0.3. Protein concentrate and processed gels scored 0 ± 0 for the characteristics studied.

4. Conclusion

An odourless functional protein concentrate was obtained from the frozen muscle of *Dosidicus gigas*. It was whiter and contained fewer impurities than surimi made from Alaska Pollack and possessed the right gel-forming capacity. The procedure consisted of the following stages: (a) partial solubilization of the muscle in 0.16 M NaCl and 0.1% $NaHCO_3$ adding an antifoaming agent; (b) filtration through an orifice of 300–400 μm to remove any remains of the connective tissue; (c) precipitation with HCl, bringing the pH of the solution to 4.7–4.9 and collecting the precipitate by centrifugation; (d) freezing with cryoprotectants and storage at −20 °C for at least 45 days. For subsequent thermal gelation at 90 °C, 0.2% $Ca(OH)_2$ had to be added to neutralize the concentrate and 1% NaCl had to be added/was added to a final content of 2%. The resulting gels had a pleasant taste and odour unlike the raw material. Better results were achieved by adding 250 ppm EDTA at the start of the processing to prevent myosin proteolysis. Two-stage gelation is not necessary since the protein is not susceptible to setting.

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