Thermal Denaturation of Muscle Proteins from Male and Female Squid (*Illex argentinus*) at Different Sexual Maturation Stages. A Differential Scanning Calorimetric Study

Maria E. Paredi,*[,†,§] Mabel C. Tomas,[‡,¶] Marcos Crupkin,[†,Δ] and Maria C. Añón[‡,¶]

Centro de Investigaciones de Tecnología Pesquera, CITEP-INTI, Marcelo T. de Alvear 1168, Mar del Plata, 7600 Buenos Aires, Argentina, and Centro de Investigaciones y Desarrollo en Criotecnología de Alimentos-CIDCA (CONICET-CIC-UNLP), Facultad de Ciencias Exactas, 47 y 116, La Plata, 1900 Buenos Aires, Argentina

The influence of both sex and sexual maturation stage of specimens on the thermal behavior of muscle proteins was investigated. Differential scanning calorimetry (DSC) thermograms of whole muscle of female squids showed four endothermic transitions [peak temperature maximum (T_{max}), 45.9, 56.8, 67.2, and 79.2 °C]. The DSC thermograms of whole muscle of male squids showed three transitions (T_{max}, 47.9, 56.8, and 79.2 °C). The T_{max} of 67.2 °C, present only in female squid muscle, was related to sarcoplasmic proteins. Myosin and paramyosin contributed to the first transition, connective tissue to the second transition, and actin to the last transition. No major differences were observed in T_{max} values that were related to the sex and sexual maturation stage of specimens. The lowest denaturation enthalpy (ΔH) was found in muscle from immature females. Independently of the sex and the sexual maturation stage of specimens, no major changes were observed in either T_{max} or ΔH values during frozen storage of squids.

Keywords: *Squid muscle; thermal stability; myofibrillar proteins; sexual maturation stage; differential scanning calorimetry*

INTRODUCTION

The study of the thermal behavior of myofibrillar proteins is of technological importance to determine and predict the final quality of meat products because functional and textural characteristics of meat mainly depend on their myofibrillar proteins. Differential scanning calorimetry (DSC) offers a direct method to study the thermal transition of muscle proteins *in situ* (Wright *et al.*, 1977). The use of thermal analysis and in particular of DSC to monitor conformational changes is relatively new in terms of food proteins. On the other hand, freezing is common in food systems; and its influence on conformational properties is therefore of interest (Arntfield *et al.*, 1990).

The thermal denaturation of myofibrillar proteins was studied in several fish species by DSC (Hastings *et al.*, 1985; Poulter *et al.*, 1985; Beas *et al.*, 1990; Howell *et al.*, 1991). Compared with the myofibrillar thermostability of several mammalian, avian, and fish species, there are few DSC reports of myofibrillar proteins of marine invertebrate species (Akahane *et al.*, 1985; Hastings *et al.*, 1985; Paredi *et al.*, 1994, 1995). Particularly scarce is information on the thermal denaturation of squid muscles and their myofibrillar proteins (Rodger *et al.*, 1984; Hastings *et al.*, 1985).

The purpose of this work was to study the influence of both sex and sexual maturation stage of squid specimens on the thermal behavior of muscle proteins by monitoring both peak temperature maxima (T_{max}) and denaturation enthalpies by DSC.

MATERIAL AND METHODS

Specimens of squid *Illex argentinus* were caught by the research ship Capitán Oca Balda (INIDEP) on the Patagonian shelf in the southwestern Atlantic between ~42° and 54° S, from February to March, 1995. Two lots of 60 fresh squids each were immediately frozen at −25 °C on board the ship and transported as frozen whole squid to the laboratory. Squids were stored at −30 °C for 9 months. The specimens were carefully thawed at 10 °C, and sex and sexual maturation stages were determined as previously described by Brunetti (1990). Only male and female squids at sexual maturation stage either 2−3 (immature) or 5 (mature) were used. Muscle from the mantle was dissected, skinned, and cut into small pieces. Portions of muscle were used for protein isolation and DSC analysis. Six samples of whole muscle were taken from both male and female squids, at either stage 2−3 or 5, at 1, 2, 5, and 9 months of frozen storage for DSC analysis.

Preparation of Muscle Proteins. The procedure followed to obtain partially purified actomyosin was previously described (Paredi *et al.*, 1990). Myofibrils were prepared by the procedure of Chantler and Szent-Gyorgyi (1980). The sarcoplasmic fraction was obtained by stirring small pieces of skinned squid muscle with a 10 mM phosphate buffer (pH 6.5) for 60 min at 2−4 °C. The suspension was strained through gauze and centrifuged at 40000*g* for 60 min. The supernatant, dialyzed during the night against the extraction buffer containing 3 mM azide, constitutes the sarcoplasmic fraction. The portion recovered from the gauze corresponds to muscle depleted of sarcoplasmic proteins.

To obtain the connective tissue, sarcoplasmic and myofibrillar proteins were extracted from the muscles with a 5 mM phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, 0.1 mM

† Centro de Investigaciones de Tecnologia Pesquera, CITEP.

§ Post-Doctoral Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

‡ Centro de Investigaciones y Desarrollo en Criotecnologia de Alimentos-CIDCA.

¶ Career Investigators of the CONICET.

Δ Professor of the Fac. Cs. Agrarias, Universidad Nacional de Mar del Plata.

S0021-8561(96)00096-9 CCC: $12.00

EGTA, and 0.6 M KCl, and the extract was centrifuged at 10 000*g* for 20 min. The supernatant containing sarcoplasmic and myofibrillar proteins was discarded, and the extraction−centrifugation cycle was repeated twice. The pellet thus obtained, which was primarily connective tissue, was used for DSC analysis. All the procedures were performed at 2−4 °C, and all the solutions contained 0.1 mM phenylmethanesulfonyl fluoride (PMSF).

Protein Determination. Protein concentration was determined on aliquots of actomyosin and sarcoplasmic protein extracts and myofibrils suspensions by the Lowry method with bovine serum albumin (BSA) as standard (Lowry *et al.*, 1951).

Criterion of Purity for Protein Preparation. The purity of proteins was assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels with a Shandon vertical gel apparatus, as reported by Portzio and Pearson (1977). The protein loaded on the gel was varied to check the linearity of the myosin heavy chain, actin, paramyosin, and myosin light chains. With 30 μg of protein, a linear response was obtained. The mobility−molecular weight curve was calibrated with standards of molecular weights, MW-SDS-70L, and albumin cross-linked bovine, from Sigma Chemical Company (St. Louis, MO).

The quantitative composition of each protein was determined by scanning the gels at 600 nm with a Shimadzu dual-wavelength chromatogram scanner (model CS 910) equipped with a gel scanning accessory (Kyoto, Japan).

Differential Scanning Calorimetry. The DSC studies were performed in a Dupont 910 system attached to a Hewlett Packard 7046 B recorder. Temperature calibrations were performed according to ASTM Norm E 474/80 using indium thermograms. The samples (20 mg wet weight) were placed in the DSC hermetic pans, assuring a good contact between the sample and the capsule bottom. Triplicate samples were analyzed. A hermetic capsule with 18 μL of distilled water was used as reference. After DSC analysis, the capsules were punctured and the dry matter weight was determined by drying at 105 °C overnight. All the samples were scanned at 10 °C/min over the range of 10 to 100 °C at a sensitivity of 0.5 mV/cm. Total denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve (a baseline was constructed as a straight line from the start to the end of the endotherm). Endotherm areas were measured with the Sigma Scan Package (The Scientific Measurement Program 3.90, 1992).

Statistical Analysis. Analysis of variance was applied to the data using the statistical analysis package SYSTAT (Wilkinson, 1990).

RESULTS AND DISCUSSION

DSC thermograms of mantle from both male and female specimens have the characteristic profiles shown in Figure 1. Because of their complexity, it is convenient to describe them in terms of their T_{max} value (Wright *et al.*, 1977). DSC thermograms of male whole muscle show three endothermic transitions with T_{max} values of 47.9 ± 0.5, 56.8 ± 0.9, and 79.3 ± 1.0 °C (Figure 1a). DSC thermograms of female whole muscle show four endothermic transitions, with T_{max} values of 45.9 ± 1.2, 56.8 ± 0.9, 67.2 ± 0.4, and 79.2 ± 0.8 °C (Figure 1b). As can be seen in Figures 1a and 1b, the DSC thermograms of female whole muscle show four transitions with one transition at 67.2 °C that is not present in male whole muscle. The ΔH values of male and female whole muscle were 20.3 ± 1.5 and 15.9 ± 1.5 *J*/g, respectively.

The DSC thermograms of mammalian whole muscles showed three transitions with T_{max} values of 57−60, 62−67, and 74−80 °C at a heating rate of 10 °C/min (Wright *et al.*, 1977; Wagner and Añón, 1986). The T_{max} values of squid mantle transitions were lower than those of

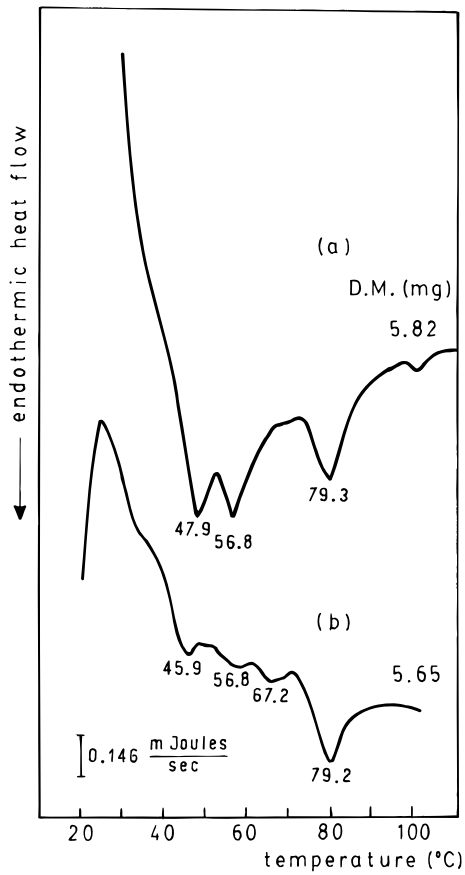

Figure 1. DSC thermograms of whole muscle of squid at sexual maturation stage 2−3 for (a) males and (b) females (heating rate, 10 °C/min; DM, dry matter).

mammalian muscles. Similar results were reported for whole muscle of other squid species (*Loligo forbesi*; Rodger *et al.*, 1984; Hastings *et al.*, 1985) and for other fish and mollusc species (Martens and Vold, 1976; Akahane *et al.*, 1985; Hastings *et al.*, 1985; Howell *et al.*, 1991; Paredi *et al.*, 1994). Connell (1961), Hasnain *et al.* (l979), Hastings *et al.* (1985), Davies *et al.* (1988), and Ogawa *et al.* (1993) attributed higher lability to fish species proteins than to mammalian muscle proteins.

The collagen content in squid muscles is ∼11% (Sikorski and Kolodziejka, 1986). Therefore, the endothermic transitions in DSC profiles of squid whole muscle would be attributed to denaturation of myofibrillar proteins, connective tissue, and sarcoplasmic proteins. The DSC profiles of whole muscle, connective tissue, and sarcoplasmic proteins from female squids are shown in Figure 2. The sarcoplasmic protein fraction contributed with transition at T_{max} values of 43.5, 49.8, 62.0, and 75 °C. One major peak at T_{max} of 56.1 ± 1.0 °C is prominent in thermograms of connective tissue. Other minor peaks are also present in these thermograms (Figure 2). The major and minor peaks would be attributed to collagen and non-collagenous proteins, respectively.

To investigate the contribution of myofibrillar proteins to DSC transitions of whole muscle, muscle depleted of sarcoplasmic protein fraction, myofibrils, and actomyosin endothermic transitions were also analyzed. The purity of myofibrils and actomyosin from squid muscle was previously checked by SDS-PAGE 10%. The characteristic polypeptide bands of the major myofibrillar proteins were present in the densitometric profiles of both myofibrils and actomyosin (Figure 3). The

Table 1. Peak Temperature (T_{max}) and Denaturation Enthalpies (ΔH_{total}) of Male and Female Squid Muscle Proteins at Different Sexual Maturation Stages[a]

sex	sexual stage	T_{max} (°C)				ΔH_{total} (J/g)
female	2−3	45.9 ± 0.9	56.8 ± 0.9	67.2 ± 0.4	79.2 ± 0.8	15.9 ± 1.5[a]
	5	44.8 ± 1.7	60.3 ± 1.0	66.7 ± 0.5	77.5 ± 0.8	19.2 ± 1.6[b]
male	2−3	47.9 ± 0.5	56.8 ± 0.9		79.3 ± 0.9	20.3 ± 1.5[b]
	5	46.0 ± 0.5	60.0 ± 1.4		79.0 ± 0.0	20.2 ± 1.3[b]

[a] Each value represents a mean of 4-6 determinations ± SD; values in the same columns with different superscripts (a and b) are significantly different ($p < 0.05$).

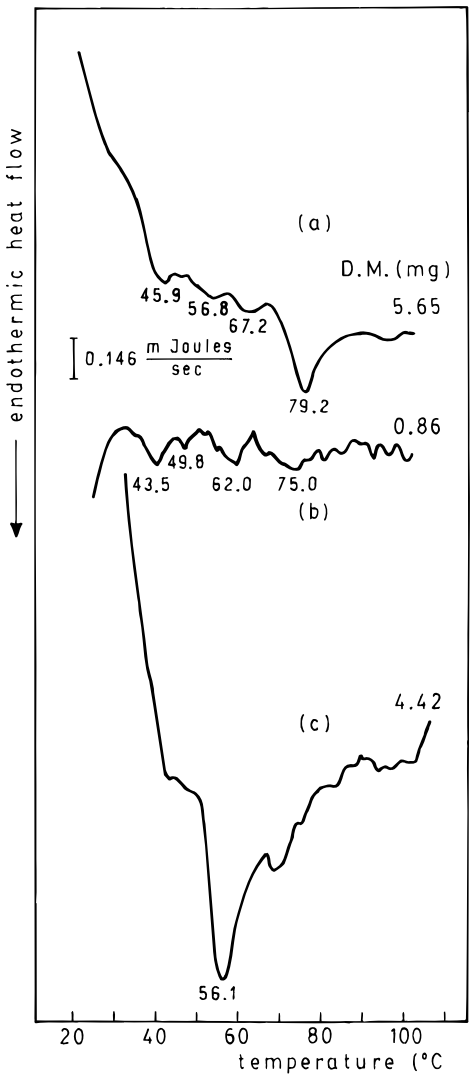

Figure 2. DSC thermograms of female squid at sexual maturation stage 2−3 in (a) whole muscle, (b) sarcoplasmic proteins, and (c) connective tissue (heating rate, 10 °C/min; DM, dry matter).

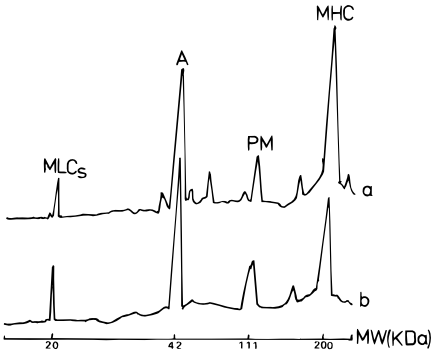

Figure 3. Densitometric patterns of SDS-PAGE 10% gels from (a) myofibrils of female squid muscle and (b) actomyosin of squid muscle: (MHC) myosin heavy chain (200 kDa); (PM) paramyosin (111 kDa); (A) actin (42 kDa); (MLCs) myosin light chains (20 kDa), and (MW) molecular weight.

female squid muscle depleted of sarcoplasmic proteins shows three endothermic transitions at 48.8, 55.5, and 77.3 °C (Figure 4). This profile is similar to the DSC thermogram obtained with whole muscle of male squid shown in Figure 1a. Therefore, the third peak observed in DSC thermograms of female muscle could be related to sarcoplasmic proteins.

The similarity of DSC thermograms obtained with either myofibrils or actomyosin is also shown in Figure 4. Two endothermic transitions were observed in both myofibrils and actomyosin, with T_{max} values of 47.8 ± 0.2 and 73.1 ± 1.4 °C for myofibrils and 45.4 ± 0.7 and 71.7 ± 1.5 °C for actomyosin. There was a displacement of the thermal transition to temperatures lower than those of whole muscle. The results shown in Figure 4 indicate a higher thermal stability for whole muscle than for isolated myofibrillar protein. These results agree with those reported by Wright *et al.* (1977), Xiong *et al.* (1987), and Paredi *et al.* (1994).

Paredi *et al.* (1994) reported two major endothermic transitions at 50.5 and 72.5 °C in whole adductor muscle from the bivalve mollusc *Aulacomya*. In that work, the first transition was related to myosin and paramyosin denaturation and the second to actin denaturation. As the densitometric analysis of SDS-PAGE 10% profiles shows, squid actomyosin has a relative composition of myosin, paramayosin, and actin similar to the actomyosin from *Aulacomya* (Figure 3, Paredi *et al.*, 1994). In this way, the first transition in DSC thermograms of both myofibrils and actomyosin from squid would be related to myosin and paramyosin denaturation and the second to actin denaturation.

The results shown in Figures 2 and 4 indicate that myosin and paramyosin contributed mainly to the first transition, connective tissue to the second transition, actin to the fourth transition, and sarcoplasmic proteins contributed to the first, third, and fourth transitions in female squid muscle. On the other hand, no major differences were observed in T_{max} values of myofibrils and actomyosin from both male and female squid muscle.

The T_{max} and ΔH values of whole muscle of male and female squids at different sexual maturation stages are shown in Table 1. No major differences in the T_{max} values were observed with either sex or sexual maturation stage of the specimens. No differences were observed in the ΔH of whole muscle of males at different sexual maturation stages.

The ΔH_{total} values of whole muscle of males were significantly higher ($p < 0.05$) than those of females when specimens at sexual maturation stage 2−3 were compared. In addition, ΔH values of whole muscle of female squids at stage 5 were significantly higher ($p <$

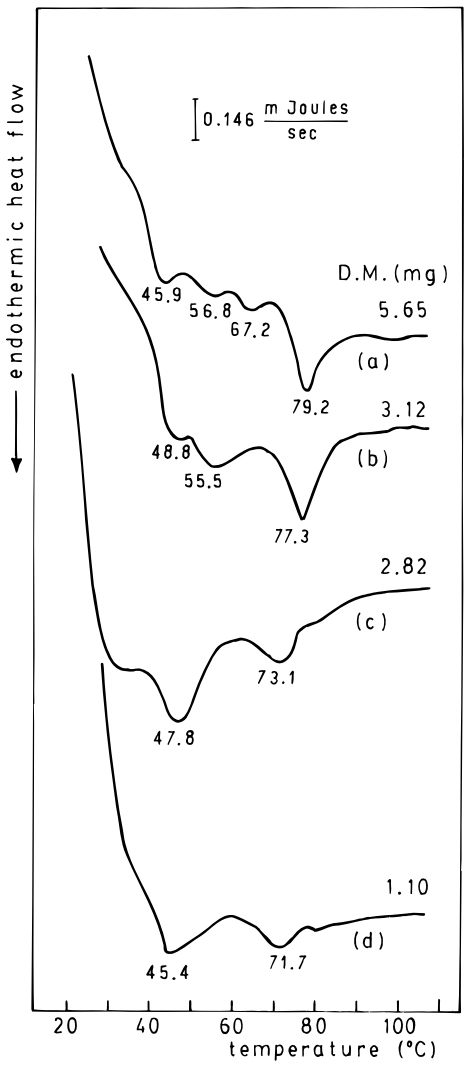

Figure 4. DSC thermograms of female squid at sexual maturation stage 2−3 in (a) whole muscle, (b) muscle depleted of sarcoplasmic proteins, (c) myofibrils, (d) actomyosin (heating rate, 10 °C/min.; DM, dry matter).

0.05) than those of females at stage 2−3. These results indicate a higher denaturation in muscle proteins of female squids at sexual maturation stage 2−3. However, more investigation is required to establish the nature of the variations in ΔH due to sex and sexual maturation stages of squid.

The effect of frozen storage on both ΔH and T_{max} of the squid muscle was also investigated. Independently of sex and sexual maturation stage of the specimens, no major changes were observed in either T_{max} or ΔH_{total} and ΔH_{peak1} during the frozen storage of squid (data not shown). Our results agree with those of Hastings *et al.* (1985) and Hsu *et al.* (1993) from DSC studies of cod muscle and Pacific whiting muscle, respectively. These researchers observed that myosin underwent some degree of partial denaturation within 2 weeks of frozen storage. As there was a negligible degree of subsequent change in myosin transition for the period following 2 weeks, the initial reduction in myosin enthalpy was attributed to the freezing process.

In conclusion, whole muscle of female squids showed four endothermic transitions during thermal denaturation and whole muscle of male squids showed three transitions. The third transition observed only in female squid muscle thermograms is attributable to sarcoplasmic proteins. The ΔH indicate the highest denaturation was observed in female squids at sexual maturation stage 2−3. No major changes were observed in either T_{max} or ΔH during frozen storage of squid.

LITERATURE CITED

Akahane, T.; Chihara, S.; Niki, T.; Sano, T.; Tsuchiya, T.; Noguchi, S. F.; Ookami, H.; Matsumoto, J. J. Differential scanning calorimetric studies on thermal behaviors of myofibrillar proteins. *Bull. Jpn. Soc. Sci. Fish.* **1985**, *51*, 1841−1846.

Arntfield, S. D.; Ismond, M. A. H.; Murray, E. D. Thermal analysis of food proteins in relation to processing effects. In *Thermal Analysis of Food*; Harwalkar, V. R., Ma, C. Y., Eds.; Elsevier Science: London, 1990; Chapter 3, pp 16−30.

Beas, V. B.; Wagner, J. R.; Crupkin, M.; Añón, M. C. Thermal denaturation of hake (*Merluccius hubbsi*) myofibrillar proteins. A differential scanning calorimetric and electrophoretic study. *J. Food Sci.* **1990**, *55*, 683−687.

Brunetti, N. E. Escala para la identificación de estadios de madurez sexual del calamar *Illex argentinus* (Castellanos, 1960). *Frente Maritimo* **1990**, *7(A)*, 45−52.

Chantler, P. D.; Szent-Gyorgyi, A. G. Regulatory light-chains and scallop myosin: Full dissociation, reversibility and cooperative effects. *J. Mol. Biol.* **1980**, *138*, 473-492.

Connell, J. J. The relative stabilities of the skeletal muscle myosins of some animals. *Biochem. J.* **1961**, *80*, 503−509.

Davies, J. R.; Bardsley, R. G.; Ledward, D. A.; Poulter, R. G. Myosin thermal stability in fish muscle. *J. Sci. Food Agric.* **1988**, *45*, 61−68.

Hasnain, A.; Samejima, K.; Yasui, T. Peculiarity of temperature sensitivity of carp and rabbit myosins. *Indian J. Exp. Biol.* **1979**, *17*, 203−206.

Hastings, R.; Rodger, G. W.; Park, R.; Matthews, A. D.; Anderson, E. M. Differential scanning calorimetry of fish muscle: the effect of processing and species variation. *J. Food Sci.* **1985**, *50*, 1503−1510.

Howell, B. K.; Matthews, A. D.; Donnelly, A. P. Thermal stability of fish myofibrils a differential scanning calorimetric study. *Int. J. Food Sci. Technol.* **1991**, *26*, 283−295.

Hsu, C. K.; Kolbe, E. R.; Morrissey, M. T.; Wang, D. Q.; MacDonald, G. A. Comparison of physical, thermal and chemical methods to measure protein denaturation in frozen pacific whiting (*Merluccius productus*). *J. Aquat. Food Prod. Technol.* **1993**, *2*(2), 31−49.

Lowry, O. H.; Rosenbrough,N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265-275.

Martens, H.; Vold, E. DSC studies of muscle tissue protein denaturation. *Proc. 22nd Eur. Meet. Res. Workers* **1976**, *19*, 3−6.

Ogawa, M.; Ehara, T.; Tamiya, T.; Tsuchiya, T. Thermal stability of fish myosin. *Comp. Biochem. Physiol.* **1993**, *106B*, 517−521.

Paredi, M. E.; De Vido de Mattio, N.; Crupkin, M. Biochemical properties of actomyosin and expressible juice of cold stored adductor muscles of *Aulacomya ater ater* (Molina). *J. Food Sci.* **1990**, *55*, 1567−1570.

Paredi, M. E.; Tomas, M. C.; Crupkin, M.; Añón, M. C. Thermal denaturation of *Aulacomya ater ater* (Molina) myofibrillar proteins: A differential scanning calorimetric study. *J. Agric. Food Chem.* **1994**, *42*, 873−877.

Paredi, M. E.; Tomas, M. C.; Crupkin, M.; Añón, M. C. Postmortem changes in adductor muscles from *Aulacomya ater ater* (Molina) stored at 2−4 °C. A differential scanning calorimetric study. *J. Agric. Food Chem.* **1995**, *43*, 1758−1761.

Portzio, M. A.; Pearson, A. Improved resolution of myofibrillar proteins with sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* **1977**, *490*, 27−34.

Poulter, R. G.; Ledward, D. A.; Godber, S; Hall, G.; Rowlands, B. Heat stability of fish muscle proteins. *J. Food Technol.* **1985**, *20*, 203−217.

Rodger, G.; Weddle, R. B.; Craig, P.; Hastings, R. Effect of alkaline protease activity on some properties of comminuted squid. *J. Food Sci.* **1984**, *49*, 117−123.

Sikorski, Z. E.; Kolodziejska, I. The composition and properties of squid. *Food Chem.* **1986**, *20*, 213−224.

Wagner, J. R.; Añón, M. C. Effect of frozen storage on protein denaturation in bovine muscle. Myofibrillar ATPase activity and differential scanning calorimetric studies. *J. Food Technol.* **1986**, *21*, 9−14.

Wilkinson, Leland. *Systat The System for Statistics*; SYSTAT: Evanston, IL, 1990.

Wright, D. J.; Leach, I. B.; Wilding, P. Differential scanning calorimetric studies of muscle and its constituent proteins. *J. Sci. Food Agric.* **1977**, *28*, 557−564.

Xiong, Y. L.; Brekke, C. J.; Leung, H. K. Thermal denaturation of muscle proteins from different species and muscle types as studied by differential scanning calorimetry. *Can. Inst. Food Sci. Technol. J.* **1987**, *20*, 357−362.

Received for review February 12, 1996. Revised manuscript received September 13, 1996. Accepted September 20, 1996.[⊗] These investigations were supported by grants of the Commission of European Communities (TS3-CT92-0109) and of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

JF960096+

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.