

# Partial Characterization of a Low-Molecular-Mass Fraction with Cryoprotectant Activity from Jumbo Squid (*Dosidicus gigas*) Mantle Muscle

Andrés Álvarez-Armenta<sup>1</sup>, Elizabeth Carvajal-Millán<sup>2</sup>, Ramón Pacheco-Aguilar<sup>1</sup>, Guillermina García-Sánchez<sup>1</sup>, Enrique Márquez-Ríos<sup>3</sup>, Susana María Scheuren-Acevedo<sup>1</sup> and Juan Carlos Ramírez-Suárez<sup>1\*</sup>

<sup>1</sup>Fishery Products Quality Laboratory, Food and Development Research Center, A.C. Carretera a La Victoria Km. 0.6, 83304 Hermosillo, Sonora, Mexico

<sup>2</sup>Biopolymers Laboratory, Food and Development Research Center, A.C. Carretera a La Victoria Km. 0.6, 83304 Hermosillo, Sonora, Mexico

<sup>3</sup>Department of Research and Postgraduate in Foods, University of Sonora, Blvd. Luis Encinas y Rosales S/N, Col. Centro, 83000 Hermosillo, Sonora, Mexico

Received: 14 May 2018

Accepted: 11 December 2018



\*Corresponding author:

Phone: +526622892400 ext: 654

Fax: +526622800421

E-mail: jcramirez@ciad.mx

ORCID IDs: 0000-0001-8907-947X (Álvarez-Armenta), 0000-0003-4390-7457 (Carvajal-Millán), 0000-0002-1669-8007 (Pacheco-Aguilar), 0000-0003-3748-0530 (García-Sánchez), 0000-0001-7850-4960 (Márquez-Ríos), 0000-0001-5801-4593 (Scheuren-Acevedo), 0000-0001-8512-1271 (Ramírez-Suárez)

## SUMMARY

Freezing conditions affect fish muscle protein functionality due to its denaturation/aggregation. However, jumbo squid (*Dosidicus gigas*) muscle protein functionality remains stable even after freezing, probably due to the presence of low-molecular-mass compounds (LMMC) as cryoprotectants. Thus, water-soluble LMMC (<1 kDa) fraction obtained from jumbo squid muscle was evaluated by Fourier transform infrared spectrometry. From its spectra, total carbohydrates, free monosaccharides, free amino acids and ammonium chloride were determined. Cryoprotectant capacity and protein cryostability conferred by LMMC were investigated by differential scanning calorimetry. Fraction partial characterization showed that the main components are free amino acids (18.84 mg/g), carbohydrates (67.1 µg/mg) such as monosaccharides (51.1 µg/mg of glucose, fucose and arabinose in total) and ammonium chloride (220.4 µg/mg). Arginine, sarcosine and taurine were the main amino acids in the fraction. LMMC, at the mass fraction present in jumbo squid muscle, lowered the water freezing point to -1.2 °C, inhibiting recrystallization at 0.66 °C. Significant myofibrillar protein stabilization by LMMC was observed after a freeze-thaw cycle compared to control (muscle after extraction of LMMC), proving the effectiveness on jumbo squid protein muscle cryostability. Osmolytes in LMMC fraction inhibited protein denaturation/aggregation and ice recrystallization, maintaining the muscle structure stable under freezing conditions. LMMC conferred protein cryostability even at the very low mass fraction in the muscle.

**Key words:** squid muscle, myofibrillar protein, monosaccharides, free amino acids, cryostability

## INTRODUCTION

Cephalopods are the fastest growing aquatic population in the world harvested in fisheries due to the increasing demand for low-cost marine species. Jumbo squid (*Dosidicus gigas*) is an endemic species to the east Pacific Ocean, and is by far the most captured squid species worldwide, representing over 1/3 of the cephalopod captures in the last years (1).

Jumbo squid studies have been of great interest in the last years, mainly because this species easily tolerates environmental variations such as of pressure, temperature and dissolved oxygen (2) making it an interesting species. There are a few species in the marine environment with the ability to migrate vertically, enduring drastic temperature and pressure changes; one of such species is *Dosidicus gigas* (jumbo squid), which is capable of living at 1200 m depths where temperature can reach 4 °C (2). Thus, it can be concluded that molecular mechanisms in jumbo squid, specifically those related to thermostability, osmoregulation and tolerance to high pressures, enable its adaptation to environmental conditions.

On the other hand, jumbo squid muscle proteins show limited technological properties, such as gelling and water retention capacities (3), even during surimi production where gel enhancers and cryoprotectants are need. Thus, different studies have been conducted in order to increase their protein functionality (3,4). However, it has been observed that jumbo squid muscle structure and protein functionality remained stable during its frozen storage (-20 °C) and even after freeze-thaw cycles (5). This stability may be attributed to the low-molecular-mass compounds (LMMC) present in the muscle of jumbo squid, product

of its adaptation to the vertical migrations. Nevertheless, no study has reported the presence of cryoprotectant compounds in cephalopods.

Thus, the main objective of the present study is to partially characterize the low-molecular-mass (<1 kDa) water-soluble fraction from jumbo squid (*D. gigas*) muscle, searching for the presence of components that can confer protection to its myofibrillar proteins during freezing.

## MATERIALS AND METHODS

### Sample preparation

Jumbo squid mantles were obtained from a local fish market in Hermosillo, Mexico. The extraction of low-molecular-mass compounds (LMMC; fraction <1 kDa) from mantle muscle of jumbo squid was carried out by homogenizing the skinned muscle in ultrapure water, obtained from a Milli-Q system (Merck Millipore Ltd., Burlington, MA, USA). Briefly, 100 g of sample were homogenized (8000 rpm, 90 s) in Milli-Q water (300 mL) with the help of a tissue homogenizer (Tekmar Tissumizer SDT 1810; IKA-Werke, Breisgau, Germany). Then, the sample was centrifuged (12 000×g, 20 min) at 4 °C in a refrigerated centrifuge (Avanti J-26S XPI; Beckman Coulter Inc., Palo Alto, CA, USA). The supernatant was ultrafiltered using a 1 kDa (nominal molecular mass limit) Amicon ultrafiltration stirred cell membrane (Merck Millipore Ltd). Finally, the permeate was transferred into a clear tube and freeze-dried using a lyophilization system (FreeZone 12; Labconco™, Kansas City, MO, USA). Thus obtained sample is considered LMMC fraction.

### Fourier transform infrared spectroscopy

In order to elucidate the composition of the lyophilized sample (LMMC, <1 kDa fraction) a FTIR spectrometer (IFS 66/S; Bruker Optics Ltd., Coventry, UK) was used. Sample was placed on a single-reflection diamond attenuated total reflectance (ATR) unit (Specac Limited, Orpington, UK) and carefully pressed down to ensure a good contact. Scans were obtained in the 4000–400 cm<sup>-1</sup> range. Analysis of the spectra was performed using the Omnic™ v. 6.1A spectra software (6). Three replicates were performed.

### Free amino acid composition

Free amino acid analysis followed the procedures described by Vázquez-Ortiz *et al.* (7) with modifications. Briefly, 95 mg of freeze-dried LMMC fraction were dissolved in 200 µL of 5 % trichloroacetic acid and centrifuged (1485×g, 15 min, 4 °C; Eppendorf® 5417R centrifuge; Eppendorf, Hamburg, Germany). Then, the supernatant was filtered using a 0.22-µm filter (Merck Millipore Ltd., Cork, Ireland) and the obtained filtrate was mixed with 200 µL of internal standard (10 µg/mL, L-α-aminobutyric acid) and 700 µL of ultrapure water. Sample derivatization was carried out by mixing 10 µL of sample with 10 µL of o-phthalaldehyde (OPA) solution: 10 mg of OPA dissolved in 250 µL of methanol with 37.5 µL of 30 % Brij™ 35 (Sigma

Diagnostics, St. Louis, MO, USA), 25 µL of 2-mercaptoethanol and gauged to 10 mL with 0.5 M borate buffer, pH=10.4. Derivatized sample was injected into a high-performance liquid chromatographer (Agilent® 1100; Agilent Technologies, Palo Alto, CA, USA) with a ZORBAX 300Extend-C18 (150 mm×4.6 mm, 3.5 µm) column. Free amino acids were eluted from the column using an elution gradient consisting of acetate buffer (pH=7.2, with 1 % tetrahydrofuran) and methanol at a flow rate of 1.2 mL/min. Amino acid detection and quantification were conducted with a fluorescence detector at 350 and 450 nm for emission and excitation, respectively. The identification of free amino acid was conducted using amino acid analytical standards (purchased from Sigma-Aldrich, Merck) as reference. The obtained chromatograms were analyzed with the ChemStation™ Rev. A.10.01 software (8).

### Sarcosine determination

Sarcosine determination in LMMC fraction was carried out using the methodology described by Bank *et al.* (9) with modifications. The sarcosine derivatization was made by mixing 200 µL of fraction with 200 µL of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) (6 mg/mL acetone). Then 600 µL of pentane was used to remove excess FMOC-Cl and hydrolysis products. Subsequently, the upper phase was removed after 30 s. The extraction process finished with the addition of 400 µL 25 % (V/V) acetonitrile in 0.25 M boric acid. Finally, 50 µL of extract were injected in the HPLC system (Agilent Technologies), equipped with a ZORBAX 300Extend-C18 (150 mm×4.6 mm, 3.5 µm) column and a fluorescence detector used at 254 and 630 nm for excitation and emission, respectively. The used mobile phase, under isocratic conditions, was as follows: solvent A: 20 mM citric acid, pH=2.85, 20 mM sodium acetate, 5 mM tetramethylammonium chloride and 0.01 % sodium azide; and solvent B: 80 % 20 mM sodium acetate, 5 mM tetramethylammonium chloride and 0.01 % sodium azide (in 20 % methanol). The obtained chromatograms were analyzed with the ChemStation™ Rev. A.10.01 software (8).

### Total carbohydrate analysis

Total carbohydrates were determined by the phenol-sulfuric acid method (10). Briefly, 65 mg of LMMC fraction were dissolved in 1 mL of ultrapure water. Then, 200 µL of this solution were taken and mixed with 200 µL of phenol reagent (5 % m/V) followed by a rapid addition of 1 mL of concentrated sulfuric acid. Colour development and cooling of the sample lasted 40 min. Finally, absorbance was measured at 490 and 480 nm for hexose and pentose quantification, respectively, using an UV-visible spectrophotometer (Cary® 50; Varian, Inc., Santa Clara, CA, USA). D-glucose and D-xylose were used as standards.

### Free monosaccharide composition

Free monosaccharides present in the LMMC fraction were analyzed by gas chromatography (GC) using the

methodology described by Rouau and Surget (11) with a few modifications. The freeze-dried sample was re-suspended in Milli-Q water at a concentration of 150 mg/mL. Then, 100  $\mu\text{L}$  of inositol (5 mg/mL  $\text{H}_2\text{O}$ ) as internal standard, 400  $\mu\text{L}$  of 2 M  $\text{H}_2\text{SO}_4$ , 500  $\mu\text{L}$  of 25 % ammonia and 1 mL of sodium borohydride (20 mg in 1 mL of dimethyl sulfoxide, DMSO) were added to 1 mL of sample solution and heated (35  $^\circ\text{C}$ , 90 min). After cooling of the sample, 100  $\mu\text{L}$  of glacial acetic acid, 2 mL of acetic anhydride and 150  $\mu\text{L}$  of methylimidazole were added and left to rest at room temperature (25  $^\circ\text{C}$ ) for 20 min. The free monosaccharide extraction was performed by mixing 6 mL of Milli-Q water and 2 mL of chloroform, left to rest for 30 s and then the water phase was removed carefully. This procedure was conducted twice by adding 8 mL of ultrapure water for each washing. Finally, the non-aqueous phase was transferred into injection vials and maintained at  $-80^\circ\text{C}$  for future analysis.

For monosaccharide determination, 5  $\mu\text{L}$  of extract were injected into a gas chromatograph system (HP 6890 GC series; Agilent, Santa Clara, CA, USA) equipped with a 225 DB (50 % cyanopropylphenyl-dimethylpolysiloxane, 30 m $\times$ 0.32 mm i.d., 0.15  $\mu\text{m}$ ) column. The chromatographic conditions were as follows: injection temperature 220  $^\circ\text{C}$ , detector temperature 260  $^\circ\text{C}$  and furnace temperature 205  $^\circ\text{C}$ , using nitrogen as the carrier gas at 1 mL/min. The obtained chromatograms were analyzed with the GC ChemStation<sup>TM</sup> Rev. A. 09.01 software (12).

#### Ammonium chloride determination

The concentration of ammonium chloride was determined by flux injection analysis (FIA)/gas diffusion technique adapted from Clinch *et al.* (13). This method determines total ammonia nitrogen content (sum of  $\text{NH}_3$  and  $\text{NH}_4^+$ ). However, the ammonium chloride in LMMC fraction solution was in the form of  $\text{NH}_4^+$ . The LMMC fraction solution (500  $\mu\text{L}$ ) was injected directly into the mobile phase (0.1 M NaOH) using the low pressure injection valve (Rheodyne 5020; Anachem, Luton, UK). The mobile phase was carried out by a Gilson MINIPLUS 2 peristaltic pump (Gilson Incorporated, Middleton, WI, USA) to a gas diffusion cell (240 mm $\times$ 1.5 mm $\times$ 0.2 mm). Finally, the produced gas ( $\text{NH}_3$ ), diffused through the membrane, was detected at 625 nm (PYE Unicam SP6-550 UV/Vis spectrophotometer; Phillips, Eindhoven, The Netherlands) by using a bromophenol blue (0.4 g/L in 0.1 M NaOH) indicator solution. A fresh standard curve using ammonium chloride in the range of 20–400  $\mu\text{mol/L}$  was prepared daily.

#### Antifreeze activity

The antifreeze activity of LMMC fraction present in the jumbo squid muscle was evaluated by differential scanning calorimetry (DSC). The antifreeze activity is determined by measuring the temperature displacement of ice recrystallization conferred by solutes present in the solution in comparison with pure water, behaviour known as thermal hysteresis. This analysis was conducted employing a calorimeter (DSC7;

Perkin Elmer de México, S.A., Monterrey, Mexico) following the method described by Lu *et al.* (14). Briefly, aliquots (approx. 40 mg) of lyophilized LMMC fraction solution (at 32.2 mg/g  $\text{H}_2\text{O}$ , solute mass fraction of LMMC present in the muscle) were transferred to stainless steel capsules and hermetically sealed. Then, capsules were gradually frozen from 20 to  $-30^\circ\text{C}$  at 1  $^\circ\text{C}/\text{min}$ . After reaching  $-30^\circ\text{C}$ , the temperature was maintained for 15 min and finally capsules were heated to 20  $^\circ\text{C}$  at 1  $^\circ\text{C}/\text{min}$ . Parameters such as crystallization point (onset temperature of crystallization transition), freezing point (onset of melting transition) and enthalpy of melting transition ( $\Delta H_m$ ) were determined from thermograms. The thermograms were analysed with the Pyris<sup>TM</sup> v. 4.02 software (15). The study was designed in order to know the interval of temperatures ( $t_h$ =holding temperatures) to ensure the presence of frozen and thawed water (partial thawing) during the unfreezing transition.

Once this thermal characterization was obtained, the sample was gradually frozen to  $-30^\circ\text{C}$  at 1  $^\circ\text{C}/\text{min}$ , maintained for 15 min, and then heated to a partially unfreezing temperature ( $t_h$ ) at 1  $^\circ\text{C}/\text{min}$ , maintained for 3 min; and finally, the sample was again frozen to  $-30^\circ\text{C}$ . The enthalpy of recrystallization ( $\Delta H_f$ ) and recrystallization point ( $t_0$ =onset of recrystallization transition) were calculated with the help of the above mentioned software. This process was repeated at different  $t_h$ . The amount of ice fraction ( $x(\%)$ ) (16) and thermal hysteresis were calculated using the following equations, respectively:

$$x(\text{ice})=1-\left(\frac{\Delta H_f}{\Delta H_m}\right)\cdot 100 \quad /1/$$

and

$$\text{Thermal hysteresis} = t_h - t_0 \quad /2/$$

#### Protein cryostability

The protein cryostability conferred by osmolytes present in the LMMC fraction was expressed as the stability of myofibrillar proteins (mainly myosin and actin) to high temperatures after being subjected to a freeze-thawing process. For this objective, a DSC study was designed using a calorimeter (DSC7; Perkin Elmer de México, S.A.). First, a myofibrillar protein concentrate (MPC) from jumbo squid muscle was obtained by homogenizing 20 g of muscle in ultrapure water (at 1:3 ratio, mantle/water) at 8000 rpm for 90 s with the help of a tissue homogenizer (Tekmar Tissumizer SDT 1810; IKA-Werke). Then, homogenate was centrifuged (12 000 $\times$ g, 20 min) at 4  $^\circ\text{C}$ , using a refrigerated centrifuge (Avanti J-26S XPI; Beckman Coulter Inc., Palo Alto, CA, USA). This process was repeated twice on the precipitate to improve the extraction of water-soluble compounds (mainly sarcoplasmic proteins and LMMC). The final precipitate was lyophilized using a freeze-dryer system (Free-Zone 12; Labconco<sup>TM</sup>). Then, lyophilized protein concentrate was re-suspended in a solution containing the LMMC fraction (at 32.2 mg/g  $\text{H}_2\text{O}$ , solute mass fraction of LMMC present in the muscle) to a final 86 % ( $m/V$ ) water content. MPC and ultrapure water (instead of LMMC fraction) were prepared and used as

control. Aliquots (approx. 40 mg) were transferred to stainless steel capsules and hermetically sealed. Capsules were analyzed by calorimetry as follows: first, the capsules were gradually frozen to  $-30\text{ }^{\circ}\text{C}$  at  $1\text{ }^{\circ}\text{C}/\text{min}$ , held at this temperature for 15 min, and then heated up to  $90\text{ }^{\circ}\text{C}$  at  $5\text{ }^{\circ}\text{C}/\text{min}$ . The temperatures of endothermic transition such as onset temperature ( $t_{\text{onset}}$ ) and maximum temperature of denaturation ( $t_{\text{max}}$ ) as well as their enthalpy of protein denaturation were observed.

### Statistical analysis

The compositional characterization is shown as a mean value  $\pm$  standard deviation (S.D.). Protein cryostability mean values were analysed with a one-way analysis of variance (ANOVA) with 5 % significance level. Tukey-Kramer test was used for multiple comparisons of mean values using the NCSS 97 software (17). All experiments were conducted in triplicate.

## RESULTS AND DISCUSSION

### Sample compositional characterization

The LMMC fraction ( $<1\text{ kDa}$  fraction) from jumbo squid muscle characterized by FTIR spectroscopy had 32.2 mg of compounds per g of muscle. Lyophilized LMMC fraction absorbed the light within the mid infrared spectrum ( $4000\text{--}500\text{ cm}^{-1}$ ) corresponding to X-H stretching ( $4000\text{--}2500\text{ cm}^{-1}$ ), double bonds ( $2000\text{--}1500\text{ cm}^{-1}$ ) and fingerprint ( $1500\text{--}600\text{ cm}^{-1}$ ) regions (Fig. 1) (18). Thus, the  $3500\text{--}2500\text{ cm}^{-1}$  spectrum indicated the presence of hydrogen bonds mainly from carbohydrates (19). However, under this same spectrum, three characteristic peaks at  $3359$ ,  $3024$  and  $2960\text{ cm}^{-1}$  were observed, which correspond to N-H (from amine groups), C-H and  $-\text{CH}_3$  stretching bonds, respectively.

Absorbance values associated with double bonds ( $2000\text{--}1500\text{ cm}^{-1}$ ), in particular carboxylic groups (C=O) and amine groups (N-H) of peptide bonds, were detected at the  $1620$  and  $1522\text{ cm}^{-1}$  regions, corresponding to amine I and amine II groups, respectively (20). Besides, amine I group showed a more intense absorbance, indicating the presence of  $\beta$ -sheet

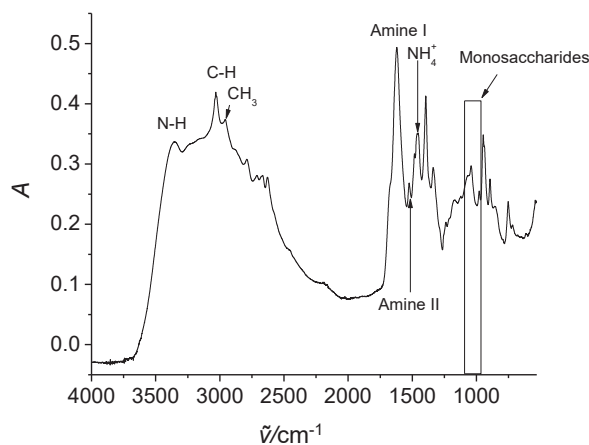


Fig. 1. FTIR spectra of low-molecular-mass (LMMC;  $<1\text{ kDa}$ ) fraction from jumbo squid muscle

peptides of low molecular mass (21) in the sample. Furthermore, there were peaks in the  $1500\text{--}600\text{ cm}^{-1}$  region related to molecular deformation (19). In this region, a peak at  $1450\text{ cm}^{-1}$  related to symmetric deformation of ammonium ion ( $\text{NH}_4^+$ ) was identified (21). This ion can be a product of ammonium chloride ( $\text{NH}_4\text{Cl}$ ) ionization, compound resulting from the catabolism of proteins and amino acids. It has been identified as the responsible for the organism's buoyancy and the sour-bitter taste of jumbo squid muscle (22,23).

Besides, inside the  $1200\text{--}1000\text{ cm}^{-1}$  region, corresponding to the deformation of monosaccharides, peaks at  $1042$  and  $1035\text{ cm}^{-1}$  indicated the stretching of the C-OH and  $\text{CH}_2\text{OH}$  bonds, respectively, both structural components of monosaccharides (24). A similar pattern was reported by Dauphin (25), who found absorbances at  $1150\text{--}1000\text{ cm}^{-1}$  in a soluble fraction of *Nautilus macromphalus* L., associated with carbohydrates. Also, absorption peaks were found within the  $1500\text{--}1300\text{ cm}^{-1}$  region, which is correlated with the presence of organic acids (24). It is important to notice that there is no research on the elucidation of the chemical composition of water-soluble extracts from jumbo squid muscle by infrared spectrophotometry. This FTIR spectroscopy analysis indicated which type of compounds to search for in the LMMC fraction in the present study.

### Total carbohydrate content

Carbohydrates found in the LMMC fraction totalled  $67.01\text{ }\mu\text{g}/\text{mg}$  of sample (Table 1). These were divided in hexoses ( $35.2\pm 5.2\text{ }\mu\text{g}/\text{mg}$ ) and pentoses ( $31.9\pm 4.6\text{ }\mu\text{g}/\text{mg}$ ). These results are similar to the values reported by Lee (26), who mentioned that carbohydrate mass fraction in cephalopods is less than 1 %. Given the presence of carbohydrates in jumbo squid muscle, it can be inferred that these compounds may influence the stability of the protein under freezing condition, as it has been observed that some carbohydrates, such as glucose and trehalose, can inhibit water crystallization (27). Besides, Baruch *et al.* (28) found that carbohydrates have potential use as cryoprotectants in freeze-thawing processes, since they observed carbohydrate antifreeze activity related to its structure and spatial disposition of the molecule hydroxyls groups in order to interact with the ice crystals.

### Free monosaccharides

Glucose, arabinose and fucose were the main free monosaccharides in the LMMC fraction from jumbo squid mantle (Table 1). The profile of free monosaccharides from jumbo squid muscle, as well as for others cephalopods species, has never been reported. Only Kani *et al.* (29) have reported the mass fraction of glucose in the muscle of four squid species (*Sepioteuthis lessoniana*, *Loligo bleekeri*, *Loligo edulis* and *Todarodes pacificus*), ranging from  $9\text{--}60\text{ mg}$  per  $100\text{ g}$  of muscle against  $100.2\text{ mg}$  per  $100\text{ g}$  of jumbo squid muscle (data not shown). This variation is probably due to different metabolic characteristics of each species.



**Table 1.** Mass fractions of total carbohydrates and free monosaccharides in low-molecular-mass compound (LMMC; <1 kDa) fraction from jumbo squid muscle

	w(total carbohydrates) µg/mg		w(free monosaccharides) µg/mg
Hexoses	35.2±5.2	Glucose	32.7±2.1
		Fucose	8.2±0.8
Pentoses	31.9±4.6	Arabinose	10.2±0.5
Total	67.1 (100 %)	Total	51.1 (76 %)

Values are the mean±standard deviation of six repetitions per sampling ( $N=3$ )

Among the identified free monosaccharides, glucose was found at a mass fraction of (32.7±2.1) µg/mg, representing 64 % of total free monosaccharides in the LMMC fraction (Table 1). Total free monosaccharides (51.1 µg/mg) represented the major constituents (76 %) of total carbohydrates (67.1 µg/mg) (Table 1) present in the fraction; the rest of carbohydrates were possibly other oligosaccharides or glycosylated biomolecules. Major free monosaccharides present in the fraction, glucose and arabinose, have been reported as cryoprotectants, stabilizing protein against its denaturation (28). Besides, glucose has been reported to confer protein stabilization and ice recrystallization inhibition during temperature fluctuations in frozen conditions (28). However, the identification of other carbohydrates such as di-, tri- and oligosaccharides, which could be present in the fraction, is needed in order to make a stronger inference about the role that carbohydrates play as protein cryostabilizers since it has been reported that di- and trisaccharides act more efficiently as osmolytes (30).

#### Free amino acid and sarcosine content

Total mass fraction of free amino acids (FAA) in LMMC was (in mg/g sample): 18.84, with arginine (8.82±0.15), sarcosine (6.95±0.76) and taurine (1.05±0.02) being the dominant FAA in the sample (Table 2). Sarcosine and taurine are characteristic amino acids of jumbo squid muscle (31), although these are not proper amino acids since both derive from them; sarcosine is an intermediary and byproduct in the synthesis and degradation of glycine, while taurine derives from cysteine (32). Ulrich (22) reported similar taurine mass fraction (10 µmol/g) in other cephalopods. Based on the study by Yancey (33), it can be suggested that the predominance of these amino acids can also contribute to the cryostability of jumbo squid proteins.

On the other hand, the protein stabilization mechanism varies among amino acids. Shukla and Trout (34) proposed that arginine interacts (cation- $\pi$  interaction) with tryptophan residues of proteins, contributing to the protein stability; however, they also proposed that arginine accumulates in the hydrophobic regions of proteins, thus inhibiting their aggregation, effect that can be replicated in jumbo squid muscle under freezing conditions.

Sarcosine has been reported as an important osmolyte stabilizer of proteins that can counteract the destabilizing effect of urea by increasing its solvation and also by its direct interaction with urea, which in turn becomes less available to interact with

**Table 2.** Free amino acid (FAA) mass fractions in low-molecular-mass compounds (LMMC; <1 kDa) from jumbo squid muscle

FAA	w/(mg/g)
Aspartic acid	0.05±0.00
Glutamic acid	0.19±0.00
Asparagine	ND
Serine	ND
Glutamine	0.09±0.00
Histidine	0.08±0.00
Arginine	8.82±0.15
Glycine	0.16±0.08
Threonine	0.13±0.00
Taurine	1.05±0.02
Alanine	0.59±0.01
Tyrosine	0.10±0.00
Methionine	0.09±0.00
Valine	0.12±0.00
Phenylalanine	ND
Isoleucine	0.10±0.04
Leucine	0.17±0.00
Lysine	0.15±0.00
Sarcosine	6.95±0.76
Total	18.84

Data are represented as average value±standard deviation ( $N=3$ ). ND=not detected. Sarcosine is not a protein amino acid, but an intermediate of glycine synthesis

proteins (35). Thus, sarcosine can show a similar protecting effect on jumbo squid proteins.

Fish protein tends to denature/aggregate under freezing conditions reducing their technological functionality; however, jumbo squid protein functionality remains stable even during freeze-thaw cycles (5), most probably due to the presence of these FAA, among other compounds.

#### Ammonium chloride content

Jumbo squid (*Dosidicus gigas*) species has an inherently high level of ammonium chloride in its muscle (23). This is an important compound that has been related to the organism's buoyancy (22) and, thus, survival. In the present study, the ammonium chloride mass fraction of the LMMC from jumbo squid muscle was (220.4±1.6) µg/mg. The presence of ammonium chloride in the LMMC can also promote jumbo squid protein muscle stability since it has been reported that some cations (including  $\text{NH}_4^+$ ) can confer stability to the native structure of proteins (36).

#### Thermal characterization

The thermal characterization of jumbo squid muscle and its LMMC fraction (32.2 mg/g) during a freeze-thawing cycle is shown in Table 3. It can be observed that both samples displaced the freezing (FP) and crystallization (CP) points towards lower temperatures than of pure water (with FP=0 °C and CP=-15 °C). LMMC fraction, at the mass fraction of 32.2 mg/g, displaced the FP of water down to -1.2 °C. The lowest displacement (down to -3.5 °C) was observed in the muscle, which was

**Table 3.** Thermal characterization of jumbo squid mantle and low-molecular-mass compound (LMMC; <1 kDa) fraction

Sample	Crystallization transition			Melting transition		
	$t_{cp}/^{\circ}\text{C}$	$\Delta H_c/(\text{J/g})$	$t_{max}/^{\circ}\text{C}$	$t_{fp}/^{\circ}\text{C}$	$\Delta H_m/(\text{J/g})$	$t_{max}/^{\circ}\text{C}$
Muscle (unwashed)	$-16.4 \pm 2.8$	$-110.2 \pm 11.1$	$-16.7 \pm 2.7$	$-3.5 \pm 0.1$	$194.9 \pm 1.8$	$1.1 \pm 0.2$
LMMC (<1 kDa) fraction*	$-14.8 \pm 1.7$	$-119.2 \pm 14.6$	$-15.4 \pm 1.8$	$-1.2 \pm 0.2$	$268.3 \pm 7.7$	$3.3 \pm 0.1$

Data are represented as average value  $\pm$  standard deviation ( $N=3$ ). \*w(LMMC extracted from muscle)=32.2 mg/g,  $t_{cp}$ =crystallization point,  $t_{fp}$ =freezing point,  $t_{max}$ =maximum temperature,  $\Delta H_c$ =enthalpy of crystallization transition,  $\Delta H_m$ =enthalpy of melting transition

to be expected due to the presence, besides the LMMC fraction, of other higher-molecular-mass compounds (*i.e.* peptides, proteins, *etc.*). Interestingly, the FP shown by the jumbo squid muscle was lower than the ones reported for other commercial fish species from the North Atlantic, which ranged from  $-1.1$  to  $-2.0$   $^{\circ}\text{C}$  (37). It is pertinent to mention that the thermal characterization (in freezing conditions) of jumbo squid muscle by DSC has not been reported elsewhere; however, Rahman and Driscoll (38) reported FP of different cephalopod species (*Sepia officinalis*, *Octopus cyanea* and *Spirula spirula*), ranging between  $-0.5$  and  $-1.7$   $^{\circ}\text{C}$ .

On the other hand, it could be observed (Table 3) that the LMMC fraction apparently did not displace the CP of water ( $-15$   $^{\circ}\text{C}$ ) towards lower temperatures, indicating that the LMMC at mass fraction of 32.2 mg/g did not confer more complexity to the aqueous medium (39). However, it is observed that higher extraction of heat is needed in that fraction ( $-119.2$  J/g vs  $-110.2$  J/g from that of muscle) for water molecules to reach a crystal structure (40). This behaviour must be mainly due to the high quantity of water available in the LMMC fraction for this process.

#### LMMC fraction antifreeze activity

The antifreeze activity analysis of the LMMC fraction is shown in Table 4. Based on a study by Mao *et al.* (41) on an insect antifreeze protein, our system can be considered to have an antifreeze capacity, showing that a delay in the onset temperature of refreezing increased from 0.39 to 0.66  $^{\circ}\text{C}$ , thus indicating that the LMMC exhibited a thermal hysteresis effect. Our result showed that the compounds present in the LMMC fraction inhibit recrystallization of water at 0.66  $^{\circ}\text{C}$ , contributing to the stabilization of muscle structure during temperature fluctuations under freezing conditions. This result is comparable with the report by Zhang *et al.* (42) about an antifreeze protein from *Avena sativa* L., whose antifreeze activity initiated at a thermal hysteresis of 0.75  $^{\circ}\text{C}$ .

**Table 4.** Thermal hysteresis of low-molecular-mass compound (LMMC; <1 kDa) fraction\* from jumbo squid muscle

$t_h/^{\circ}\text{C}$	$t_o/^{\circ}\text{C}$	$x(\text{ice})/\%$	Thermal hysteresis/ $^{\circ}\text{C}$
0.11	$-0.02$	50.35	0.13
0.34	0.22	19.24	0.12
0.53	0.41	9.42	0.12
0.73	0.34	9.66	0.39
0.93	0.27	0.90	0.66

\*w(LMMC extracted from muscle)=32.2 mg/g,  $t_h$ =holding temperature,  $t_o$ =recrystallization point,  $x(\text{ice})=1-(\Delta H_c/\Delta H_m) \cdot 100$ , where  $\Delta H_c$ =enthalpy of crystallization transition and  $\Delta H_m$ =enthalpy of melting transition

The thermal behaviour of these LMMC present in the <1 kDa fraction should be due to their effect on the colligative water properties; however, Yeh and Feeny (43) mentioned that if a system shows thermal hysteresis during a recrystallization process, it is not due to colligative mechanisms but to compounds interacting with the ice crystal surface, thus avoiding the unfrozen water to reincorporate into their surface (evading recrystallization).

It is true that the antifreeze activity, expressed as thermal hysteresis, cannot only be ascribed to monosaccharides and other low-molecular-mass compounds in the fraction because there are reports of other oligosaccharides (*i.e.* lactose, trehalose and stachyose, among others) (28) and low-molecular-mass proteins/peptides (not quantified in the present study) conferring antifreeze activity; however, results shown in the present study could elucidate this behaviour. Besides, further characterization of the LMMC fraction is necessary to clarify which other low-molecular-mass compounds, besides the ones shown in the present study, confer cryoprotection to jumbo squid muscle.

The antifreeze activity conferred by the LMMC fraction is important for the jumbo squid processing and distribution industry, since its compounds can avoid the recrystallization/crystal growing of ice during temperature fluctuations at frozen storage, thus evading the rupture of cell membranes and muscular structure changes responsible for the syneresis during the thawing process and therefore, the loss of nutrients, mass and technological properties.

#### Myofibrillar protein cryostability

The jumbo squid myofibrillar protein cryostability (mainly myosin and actin proteins) was related to the thermal changes in their denaturation transitions (Table 5). The obtained thermal behaviour was similar to the one reported by García-Sánchez *et al.* (5). The thermograms of samples showed two main endothermic transition peaks, one around 40–50  $^{\circ}\text{C}$  and the other between 70 and 80  $^{\circ}\text{C}$ , corresponding to myosin and actin proteins, respectively (data not shown).

Table 5 shows that control treatment (myofibrillar concentrate without the LMMC fraction) reduced  $t_{onset}$  and  $t_{max}$  significantly ( $p<0.05$ ) when comparing them with the muscle (unwashed) and the MPC with the LMMC fraction. On the other hand, these treatments (unwashed muscle and MPC with the LMMC fraction) showed similar ( $p \geq 0.05$ )  $t_{onset}$  and  $t_{max}$ , indicating the effect of the LMMC (among other compounds in that fraction and in the muscle) on the myosin stability of jumbo squid muscle during the frozen storage.

**Table 5.** Myofibrillar protein cryostability conferred by the low-molecular-mass compound (<1 kDa) fraction\* from jumbo squid muscle

Sample	Transition of myosin denaturation			Transition of actin denaturation		
	$t_{\text{onset}}/^{\circ}\text{C}$	$\Delta H/(\text{J/g})$	$t_{\text{max}}/^{\circ}\text{C}$	$t_{\text{onset}}/^{\circ}\text{C}$	$\Delta H/(\text{J/g})$	$t_{\text{max}}/^{\circ}\text{C}$
Muscle (unwashed)	(44.2±0.1) <sup>a</sup>	(0.12±0.01) <sup>a</sup>	(46.4±0.3) <sup>a</sup>	(73.4±1.8) <sup>a</sup>	(0.35±0.14) <sup>a</sup>	(77.2±0.6) <sup>a</sup>
MPC+LMMC	(44.8±0.0) <sup>a</sup>	(0.11±0.09) <sup>a</sup>	(46.9±0.4) <sup>a</sup>	(74.1±0.1) <sup>a</sup>	(0.34±0.18) <sup>a</sup>	(75.7±0.1) <sup>b</sup>
MPC+H <sub>2</sub> O (control)	(39.7±1.3) <sup>b</sup>	(0.08±0.03) <sup>a</sup>	(43.6±0.4) <sup>b</sup>	(69.6±0.7) <sup>a</sup>	(0.20±0.02) <sup>a</sup>	(72.8±0.9) <sup>c</sup>

Values in columns with different letters in superscript are statistically different ( $p < 0.05$ ), \*w(LMMC extracted from muscle)=32.2 mg/g,  $\Delta H$ =enthalpy of transition,  $t_{\text{max}}$ =maximum temperature, MPC=myofibrillar protein concentrate

With respect to the enthalpy of denaturation ( $\Delta H$ ) of myosin protein, all samples needed similar amount of heat ( $p \geq 0.05$ ) to denature this protein. However, MPC without the LMMC fraction showed the lowest value (Table 5), indicating the relationship of LMMC fraction and the freezing stability of protein.

On the other hand, jumbo squid actin in MPC without the LMMC fraction had the lowest ( $p < 0.05$ ) values of  $t_{\text{onset}}$  and  $t_{\text{max}}$  (69.6 and 72.8 °C, respectively) of all samples (Table 5), showing the effect of that fraction on the protein stability of jumbo squid muscle during frozen storage. Actin showed a similar  $\Delta H$  tendency as myosin, decreasing its value ( $p \geq 0.05$ ) as the treatments with/without the LMMC fraction were applied. From these last results, it is evident that the MPC system, in the absence of the LMMC fraction, had lower stability (at frozen storage) than the systems with that fraction.

These results indicate that myofibrillar proteins (at frozen storage) are stabilized by osmolytes present in the LMMC fraction, such as arginine and sarcosine, both of which have been reported to show a high protein-stabilizing effect (44). However, more research is necessary to completely elucidate the stabilizing effect during freezing of these compounds on myofibrillar proteins and conduct a thorough compositional characterization of the LMMC fraction since the effect of other compounds (*i.e.* trimethylamine oxide and betaine) on protein stability has also been reported (45).

To summarise, results of the present study confirm the relevance of not washing the jumbo squid muscle, for example during the surimi production, since it only takes away compounds responsible for the muscle's cryostability. Besides, after extensively washing the muscle, cryoprotectants (mainly sucrose and sorbitol) need to be added. Therefore, applying washing and adding cryoprotectants only increases the cost of surimi production. Instead, it is recommended to freeze the jumbo squid mantle muscle as such for further direct use as a raw material for fish analogues. However, if removal of the common sour-acidic squid flavour is required, washing the defrosted mantles is an alternative.

## CONCLUSION

The results lead to the conclusion that the low-molecular-mass compound (LMMC) fraction from jumbo squid mantle muscle, composed of osmolytes such as monosaccharides and free amino acids, protects the species myofibrillar proteins from freezing denaturation/aggregation, thus maintaining

their technological functionality. It is well known that the surimi industry uses some of these LMMC because they can confer cryostability to proteins; however, so far no study has shown their effect (at the mass fraction present in the muscle, which is well below the one used by the industry) on the thermal properties of water, as well as on the protein muscle cryostability.

## ACKNOWLEDGEMENTS

Author AAA wishes to thank Consejo Nacional de Ciencia y Tecnología of México for the scholarship received during the performing of this research. Authors want to thank M.S. Karla Gpe. Martínez Robinson for her help in the study.

## REFERENCES

- Markaida U, Gilly WF. Cephalopods of Pacific Latin America. *Fish Res.* 2016;173:113–21. <https://doi.org/10.1016/j.fishres.2015.09.014>
- Stewart JS, Field JC, Markaida U, Gilly WF. Behavioral ecology of jumbo squid (*Dosidicus gigas*) in relation to oxygen minimum zones. *Deep Sea Res Part II Top Stud Oceanogr.* 2013;95:197–208. <https://doi.org/10.1016/j.dsr2.2012.06.005>
- Cortés-Ruiz JA, Pacheco-Aguilar R, Lugo-Sánchez ME, Carvallo-Ruiz GM, García-Sánchez G. Production and functional evaluation of a protein concentrate from giant squid (*Dosidicus gigas*) by acid dissolution and isoelectric precipitation. *Food Chem.* 2008;110(2):486–92. <https://doi.org/10.1016/j.foodchem.2008.02.030>
- Ramírez-Suarez JC, Álvarez-Armenta A, García-Sánchez G, Pacheco-Aguilar R, Scheuren-Acevedo SM, Mazorra-Manzano MA, et al. Effect of amidated low-methoxyl pectin on physicochemical characteristics of jumbo squid (*Dosidicus gigas*) mantle muscle gels. *Food Technol Biotechnol.* 2017;55(3):398–404. <https://doi.org/10.17113/ftb.55.03.17.5001>
- García-Sánchez G, Sotelo-Romero CR, Pacheco-Aguilar R, Ramírez-Suárez JC, Sotelo-Mundo R, Scheuren-Acevedo SM, et al. Effect of freezing on protein denaturation and gelling capacity of jumbo squid (*Dosidicus gigas*) mantle muscle. *LWT – Food Sci Technol.* 2015;60(2):737–42. <https://doi.org/10.1016/j.lwt.2014.10.051>
- Omnic™, v. 6.1A, Thermo Nicolet Cooperation, Madison, WI, USA; 2001. Available from: <https://www.thermofisher.com/>.

7. Vázquez-Ortíz FA, Caire G, Higuera-Ciajara I, Hernández G. High performance liquid chromatographic determination of free aminoacids in shrimp. *J Liq Chromatogr.* 1995; 18(10):2059–68.  
<https://doi.org/10.1080/10826079508013960>
8. ChemStation™, Rev. A.10.01, Agilent Technologies, Inc., Waldbronn, Germany; 2003. Available from: <https://www.agilent.com/>.
9. Bank RA, Jansen EJ, Beekman B, te Koppele JM. Amino acid analysis by reverse-phase high-performance liquid chromatography: Improved derivatization and detection conditions with 9-fluorenylmethyl chloroformate. *Anal Biochem.* 1996;240(2):167–76.  
<https://doi.org/10.1006/abio.1996.0346>
10. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Biochem.* 1956;28(3):350–6.  
<https://doi.org/10.1021/ac60111a017>
11. Rouau X, Surget A. A rapid semi-automated method for the determination of total and water-extractable pentosans in wheat flours. *Carbohydr Polym.* 1994;24(2):123–32.  
[https://doi.org/10.1016/0144-8617\(94\)90022-1](https://doi.org/10.1016/0144-8617(94)90022-1)
12. GC ChemStation™, Rev. A.09.01, Agilent Technologies, Inc., Waldbronn, Germany; 2001. Available from: <https://www.agilent.com/>.
13. Clinch JR, Worsfold PJ, Casey H. An automated spectrophotometric field monitor for water quality parameters: Determination of nitrate. *Anal Chim Acta.* 1987;200:523–31.  
[https://doi.org/10.1016/S0003-2670\(00\)83797-6](https://doi.org/10.1016/S0003-2670(00)83797-6)
14. Lu M, Wang B, Li Z, Fei Y, Wei L, Gao S. Differential scanning calorimetric and circular dichroistic studies on plant antifreeze proteins. *J Therm Anal Calorim.* 2002;67(3):689–98.  
<https://doi.org/10.1023/A:1014369208229>
15. Pyris™, v. 4.02, PerkinElmer, Inc., Waltham, MA, USA; 2001. Available from: <https://www.perkinelmer.com/>.
16. Zhang C, Zhang H, Wang L, Yao H. Validation of antifreeze properties of glutathione based on its thermodynamic characteristics and protection of baker's yeast during cryopreservation. *J Agric Food Chem.* 2007;55(12):4698–703.  
<https://doi.org/10.1021/jf070387q>
17. NCSS 97, NCSS, LLC, Kaysville, UT, USA; 1997. Available from: <https://www.ncss.com/>.
18. Stuart B. Infrared spectroscopy: Fundamentals and applications. In: Ando DJ, editor. *Analytical techniques in the sciences.* Chichester, UK: John Wiley & Sons, Ltd.; 2004.  
<https://doi.org/10.1002/0470011149>
19. Barth A, Zscherp C. What vibrations tell us about proteins. *Q Rev Biophys.* 2002;35(4):369–430.  
<https://doi.org/10.1017/S0033583502003815>
20. Miller LM, Bourassa MW, Smith RJ. FTIR spectroscopic imaging of protein aggregation in living cells. *BBA – Biomembranes.* 2013;1828(10):2339–46.  
<https://doi.org/10.1016/j.bbamem.2013.01.014>
21. Max JJ, Chapados C. Aqueous ammonia and ammonium chloride hydrates: Principal infrared spectra. *J Mol Struct.* 2013;1046:124–35.  
<https://doi.org/10.1016/j.molstruc.2013.04.045>
22. Urich K. *Comparative animal biochemistry.* Berlin, Germany: Springer; 1994.  
<https://doi.org/10.1007/978-3-662-06303-3>
23. Márquez-Ríos E, Morán-Palacio EF, Lugo-Sánchez ME, Ocano-Higuera VM, Pacheco-Aguilar R. Postmortem biochemical behavior of giant squid (*Dosidicus gigas*) mantle muscle stored in ice and its relation with quality parameters. *J Food Sci.* 2007;72(7):356–62.  
<https://doi.org/10.1111/j.1750-3841.2007.00468.x>
24. D'Souza L, Devi P, Divya Shridhar MP, Naik CG. Use of Fourier transform infrared (FTIR) spectroscopy to study cadmium-induced changes in *Padina tetrastratica* (Hauck). *Anal Chem Insights.* 2008;3:135–43.  
<https://doi.org/10.4137/117739010800300001>
25. Dauphin Y. Structure and composition of the septal nacreous layer of *Nautilus macromphalus* L. (Mollusca, Cephalopoda). *Zoology.* 2006;109(2):85–95.  
<https://doi.org/10.1016/j.zool.2005.08.005>
26. Lee PG. Nutrition of cephalopods: Fueling the system. *Mar Freshw Behav Phys.* 1995;25(1-3):35–51.  
<https://doi.org/10.1080/10236249409378906>
27. Gonda T, Sei T. The inhibitory growth mechanism of saccharides on the growth of ice crystals from aqueous solutions. *Prog Cryst Growth Charact Mater.* 2005;51(1-3):70–80.  
<https://doi.org/10.1016/j.pcrysgrow.2005.10.005>
28. Baruch E, Belostotskii AM, Mastai Y. Relationship between the antifreeze activities and the chemical structures of polyols. *J Mol Struct.* 2008;874(1-3):170–7.  
<https://doi.org/10.1016/j.molstruc.2007.03.054>
29. Kani Y, Yoshikawa N, Okada S, Abe H. Comparison of extractive components in muscle and liver of three Loliginidae squids with those of one Ommastrephidae species. *Fish Sci.* 2007;73: 940–9.  
<https://doi.org/10.1111/j.1444-2906.2007.01417.x>
30. Poddar NK, Ansari ZA, Singh RK, Moosavi-Movahedi AA, Ahmad F. Effect of monomeric and oligomeric sugar osmolytes on  $\Delta G_D$ , the Gibbs energy of stabilization of the protein at different pH values: Is the sum effect of monosaccharide individually additive in a mixture? *Biophys Chem.* 2008;138(3):120–9.  
<https://doi.org/10.1016/j.bpc.2008.09.013>
31. Rosas-Romero ZG, Ramírez-Suarez JC, Pacheco-Aguilar R, Lugo-Sánchez ME, Carvallo-Ruiz G, García-Sánchez G. Partial characterization of an effluent produced by cooking of jumbo squid (*Dosidicus gigas*) mantle muscle. *Bioresour Technol.* 2010;101(2):600–5.  
<https://doi.org/10.1016/j.biortech.2009.08.074>



32. Hanley MR. The identification of dansyl sarcosine and its occurrence in molluscs. *Experientia*. 1975;31(8):881–3. <https://doi.org/10.1007/BF02358827>
33. Yancey PH. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol*. 2005;208(15):2819–30. <https://doi.org/10.1242/jeb.01730>
34. Shukla D, Trout BL. Interaction of arginine with proteins and the mechanism by which it inhibits aggregation. *J Phys Chem B*. 2010;114(42):13426–38. <https://doi.org/10.1021/jp108399g>
35. Kumar N, Kishore N. Structure and effect of sarcosine on water and urea by using molecular dynamics simulations: Implications in protein stabilization. *Biophys Chem*. 2013; 171:9–15. <https://doi.org/10.1016/j.bpc.2012.11.004>
36. Zhao H. Effect of ions and other compatible solutes on enzyme activity, and its implication for biocatalysis using ionic liquids. *J Mol Catal B: Enzym*. 2005;37(1-6):16–25. <https://doi.org/10.1016/j.jmolcatb.2005.08.007>
37. Tolstorebrov I, Eikevik TM, Bantle M. A DSC study of phase transition in muscle and oil of the main commercial fish species from the North-Atlantic. *Food Res Int*. 2014;55:303–10. <https://doi.org/10.1016/j.foodres.2013.11.026>
38. Rahman MS, Driscoll RH. Freezing points of selected seafoods (invertebrates). *Int J Food Sci Tech*. 1994;29(1):51–61. <https://doi.org/10.1111/j.1365-2621.1994.tb02046.x>
39. Rahman MS, Machado-Velasco M, Sosa-Morales ME, Velez-Ruiz JF. Freezing point: measurement, data, and prediction. In: Rahman MS, editor. *Food properties handbook*. Boca Raton, FL, USA: CRC press; 2009. pp. 153–92.
40. Jouppila K, Roos YH. Crystallization: measurements, data, and prediction. In: Rahman MS, editor. *Food properties handbook*. Florida, USA: CRC press; 2009. pp. 323–45.
41. Mao X, Liu Z, Li H, Ma J, Zhang F. Calorimetric studies on an insect antifreeze protein ApAFP752 from *Anatolica polita*. *J Therm Anal Calorim*. 2011;104(1);343–9. <https://doi.org/10.1007/s10973-010-1067-3>
42. Zhang YJ, Zhang H, Ding XL, Cheng LL, Wang L, Qian HF, et al. Purification and identification of antifreeze protein from cold-acclimated oat (*Avena sativa* L.) and the cryoprotective activities in ice cream. *Food Bioprocess Technol*. 2016;9(10):1746–55. <https://doi.org/10.1007/s11947-016-1750-x>
43. Yeh Y, Feeney RE. Antifreeze proteins: structures and mechanisms of function. *Chem Rev*. 1996;96(2):601–18. <https://doi.org/10.1021/cr950260c>
44. Chaudhuri P, Rashid N, Thapliyal C. Osmolyte system and its biological significance. In: Singh LR, Dar TA, editors. *Cellular osmolytes – From chaperoning protein folding to clinical perspective*. Singapore, Singapore: Springer; 2017. pp. 1–34. [https://doi.org/10.1007/978-981-10-3707-8\\_1](https://doi.org/10.1007/978-981-10-3707-8_1)
45. Chhabra G, Chandra N, Swaminathan R. Osmolytes: key players in regulating protein aggregation. In: Singh LR, Dar TA, editors. *Cellular osmolytes – From chaperoning protein folding to clinical perspective*. Singapore, Singapore: Springer; 2017. pp. 97–119. [https://doi.org/10.1007/978-981-10-3707-8\\_5](https://doi.org/10.1007/978-981-10-3707-8_5)