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Cryoprotective effect of low-molecular-mass nitrogen compounds on the myofibrillar protein of the jumbo squid (*Dosidicus gigas*) muscle

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Abstract

The study aimed to evaluate the low molecular-mass nitrogen compounds (LMMNC) arginine (Arg), taurine (Tau), and trimethylamine oxide (TMAO) (individually or their mixtures) cryostabilizing effect over jumbo squid (*Dosidicus gigas*) myofibrillar protein (JSMP) frozen at -20°C for 0, 30, and 90 days. Washed muscle (three times, 1:3, muscle:water) was lyophilized (sample). Washing water was ultrafiltrated (< 1 kDa cut-off), and permeate was lyophilized (< 1 kDa fraction). LMMNC, Control+ (with < 1 kDa fraction)] added at muscle concentration, and control (washed-muscle only) were stored at -20°C. The cryoprotective effect on JSMP was evaluated by differential scanning calorimetry, surface hydrophobicity (SoANS), solubility, and total sulfhydryl (TS) content. Myosin thermal stability increased ($P \leq 0.05$) in Control+ (43.6 ± 0.1), TMAO (44.3 ± 0.3), and Arg + TMAO (44.2 ± 0.9) compared with Con (42.6 ± 0.3) before freezing (day 0). At 90 days of storage, all TMAO-involved treatments and Control+ remained higher ($p \leq 0.05$) than Control. Solubility increased ($p \leq 0.05$) in Tau (11.2 ± 0.0 to 12.8 ± 0.1) and Arg+Tau (10.6 ± 0.1 to 12.7 ± 0.0) comparing day 0 *vs*. 90, respectively. LMMNC reduced SoANS ($p \leq 0.05$, Arg+Tau+TMAO) throughout storage. LMMNC reduced ($p > 0.05$) TS in most treatments. LMMNC, especially Taurine and TMAO, protects JSMP from freezing denaturation, stabilizing its structure.

Keywords: jumbo squid; cryoprotecting effect; low molecular-mass nitrogen compounds.

Practical Application: Low-molecular-mass nitrogen compounds function as cryoprotectants for myofibrillar proteins.

1 INTRODUCTION

Jumbo squid (JS) has been of great interest because it easily tolerates environmental variations in pressure, dissolved oxygen, and temperature since it migrates from the surface, where it feeds, to > 1,300 m deep, where temperatures reach 2–4°C (Hawkins & Storey, 2020; Stewart et al., 2013). This tolerance suggests that the JS has molecular mechanisms of environmental adaptation, specifically related to the cryostability of its muscle proteins, their body osmoregulation, and tolerance to high pressures, which would have broad technological implications related to its post-capture management and the generation of manufactured products that involve the use of freezing treatments.

On the contrary, studies have shown that JS myofibrillar proteins (JSMPs) are stable under freeze–thaw conditions (García-Sánchez et al., 2015), suggesting that this cryostability can be attributed to synthesized and accumulated compounds in the muscle because of its adaptation to its vertical migration. In addition to being involved in the JSMP stability due to environmental changes, these compounds can inhibit their deterioration when the muscle is subjected to freeze–thaw cycles during handling and post-capture processing, guaranteeing its technological functionality. Regarding this, it has been shown that the presence of low-molecular-mass compounds (< 1 kDa) in the water-soluble fraction (WSF) obtained from JS muscle had a significant myofibrillar protein stabilization after a freeze–thaw cycle. Osmolytes in this fraction inhibited protein denaturation/aggregation and ice recrystallization, maintaining the muscle structure stable under freezing conditions. The same study identified the presence of free amino acids and carbohydrates, among other compounds in the fraction (Álvarez-Armenta et al., 2019). Among osmolytes found in the < 1 kDa fraction, low molecular-mass nitrogen compounds (LMMNC) such as arginine, taurine, and trimethylamine oxide (TMAO) were among the most abundant in the characterized fraction. Thus, the objective

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of this study was to evaluate the cryostabilizing effect of arginine (Arg), taurine (Tau), and TMAO, individually or their mixtures, over JSMP after frozen storage (-20°C) for 0, 30, and 90 days.

2 MATERIALS AND METHODS

2.1 Sample preparation

A myofibrillar protein concentrate (MPC) and the WSF (< 1 kDa) were obtained from JS muscle as in Álvarez-Armenta et al. (2019). Then, MPC was reconstituted to a final 86% water content with Arginine (0.284 mg/g), Taurine (0.034 mg/g), and TMAO (13.1 mg/g), with double and triple combinations, added at a concentration found in JS muscle. MPC with WSF added (at 32.2 mg/g H_2O, solute mass fraction of osmolytes in the muscle) was used as a positive control (Con+). Then, samples were frozen at -20°C for 0 (no freezing), 30, and 90 days. Washed muscle (without < 1 kDa fraction) was used as a control (Con). Samples were thawed at room temperature for their analyses.

2.2 Protein solubility analysis

To evaluate the changes in the solubility of the MPC samples with/without the different osmolytes due to their protein aggregation during freezing storage, the methodology proposed by Hsu and Ko (2001) was used. Briefly, 2 g of sample was homogenized in 18 mL of 20 mM phosphate buffer, 0.6 M NaCl (pH 7), with a tissue homogenizer (model TR-10, Tekmar Tissumizer SDT 1810; IKA-Werke, Breisgau, Germany) at 12,000 rpm for 2 min. The homogenate was centrifuged at 20,000 × g for 15 min at 4°C in a refrigerated centrifuge (Avanti J-26S XPI; Beckman Coulter Inc., Palo Alto, CA, USA). The protein concentration was determined with biuret before and after centrifugation. Protein solubility was obtained as follows (Equation 1):

$$\text{Protein solubility (\%)} = (A/B) \times 100 \quad (1)$$

where:

A = protein concentration before centrifugation;

B = protein concentration in the supernatant after centrifugation.

Analysis was done in triplicate on each sampling day.

2.3 Differential scanning calorimetry of samples

To observe the effect of frozen storage on the myofibrillar protein of samples, a differential scanning calorimetric (DSC) study was used as in Álvarez-Armenta et al. (2019) except that the heating process was conducted at 1°C/min.

2.4 Protein surface hydrophobicity analysis

The protein surface hydrophobicity of frozen/thaw samples was quantified with the method described by Benjakul et al. (1997), as follows: An aliquot of the soluble myofibrillar supernatant obtained in the protein solubility analysis was diluted to 1 mg/mL. Subsequently, 2 mL was mixed with 10 µL of 8 mM of the hydrophobic fluorescence probe 1-anilinonaphthalene-8-sulfonic acid (ANS) solution, stirred for 10 s in a vortex, and left still for 15 min. The exact process was carried out using a standard bovine serum albumin (BSA) protein. Afterward, readings were taken in a fluorometer (Spectra Max M3 microplate reader) at excitation and emission wavelengths of 374 and 470 nm, respectively. The initial slope of the plot of fluorescence intensity *vs*. BSA protein concentration was referred to as SoANS. Its calculation was as follows (Equation 2):

$$SoANS = \frac{SoANS\ (X)}{SoANS\ (BSA)}, \quad (2)$$

where:

SoANS: relative hydrophobicity to BSA;

SoANS (X): sample hydrophobicity;

SoANS (BSA): BSA hydrophobicity.

2.5 Total sulfhydryl changes

The total sulfhydryl (TS) content on frozen samples was determined using the Ellman's reagent 5,5´-dithio-bis(2-nitrobenzoic acid) (DTNB) as described by Hamada et al. (1994) with a slight modification (Ramirez-Suarez & Morrissey, 2006). The supernatant obtained during soluble protein analysis was diluted to 0.5 mg/mL protein concentration with 20 mM phosphate buffer/0.6 M NaCl (pH 7.0). The diluted sample (0.5 mL) was mixed with 2 mL of 8 M urea in 0.2 M Tris–HCl buffer (pH 7.0) and 50 µL of 10 mM DTNB solution. Samples were incubated at 40°C for 15 min before measuring the absorbance at 412 nm. The SH content was calculated based on absorbance using the molar extinction coefficient of 13,600 $M^{-1} \bullet cm^{-1}$ (Riddles et al., 1979).

2.6 Statistical analysis

Results are shown as a mean value ± standard deviation (SD). Data were analyzed with a one-way analysis of variance (ANOVA) with 5% significance level ($p \leq 0.05$). The Tukey-Kramer test was used for multiple comparisons of mean values using the NCSS 2023 software. Two samplings were conducted, and all experiments were done in triplicate.

3 RESULTS AND DISCUSSION

3.1 Solubility of JSMP

Preserving fish muscle freshness is vital for the processing industry because it is highly susceptible to spoilage and deterioration. Freezing technology is the method most used to preserve fish muscle freshness. However, this preservation method (and everything that comes with its use, i.e., freezing rate, storage, thawing, etc.) can be deleterious, producing quality changes in fish muscle, affecting the functionality of its proteins, owing to its denaturation-aggregation (Nakazawa & Okazaki, 2020). Thus, cryoprotectants must be used to prevent this detrimental effect on protein functionality in aquatic species.

As stated before, JSMP has shown some stability in freeze–thaw conditions (Álvarez-Armenta et al., 2019; García-Sánchez et al., 2015); besides, a study in our laboratory showed that osmolytes present in its < 1 kDa WSF are (at least in part) responsible for its protein cryoprotection (Álvarez-Armenta et al., 2019).

Table 1 shows the effect of osmolytes on JSMP solubility after 90 days of frozen storage (-20°C). Osmolytes showed different effects on myofibrillar solubilization as different values ($p \leq 0.05$) were observed depending on the osmolyte used, ranging from 9.5% for Arg+Tau to 12.9% for TMAO and Arg treatments. Frozen storage resulted in detrimental for the Control sample (MPC without any osmolyte or WSF) as its solubilization diminished ($p \leq 0.05$) as time elapsed at these conditions. On the contrary, adding the WSF to the MPC (Con+) stabilized ($p > 0.05$) their protein during the frozen time. These results were expected as it has been shown that WSF preserves protein from frozen denaturation (Álvarez-Armenta et al., 2019).

When comparing day 0 *vs*. day 90 of frozen storage, most treatments were unaffected by the frozen conditions ($p > 0.05$), indicating the cryoprotective effect of these osmolytes over the protein. Besides, Tau and Arg+Tau treatments resulted in higher ($p \leq 0.05$) solubilities, 11.2 ± 0.0 *vs*.12.8 ± 0.1 and 10.6 ± 0.1 *vs*. 12.7 ± 0.0, respectively. Taurine, the osmolyte common in both treatments, is a cryoprotectant in other mollusks such as *Mytilus edulis* (Loomis et al., 1988) and also a natural osmolyte that keeps the gastropod *Nucella lima* from freezing during the cold winters in Alaska (Stickle et al., 2010).

3.2 Effect of LMMC on the calorimetry of JSMP

DSC of JSMP showed three endothermic peaks with maximum temperature (T_{max}) of denaturation at 41.1–45°C (myosin), 57.4–61.3°C, and 68.9–73.6°C (actin). However, we will only focus on the myosin transition because it is primarily responsible for the functionality of the muscle proteins. Table 2 shows the JS (*D. gigas*) myosin cryostability (T_{max}, °C) conferred by different osmolytes after 90 days of frozen storage (-20°C). Myosin thermal stability increased ($p \leq 0.05$) in TMAO (44.3 ± 03°C) and Arg+TMAO (44.2 ± 0.9°C) treatments when comparing them against control (42.6 ± 0.3°C) at day 0. Besides the frozen storage treatments, we must mention that they were frozen during the DSC analysis, so osmolytes worked as cryoprotectants. Besides, all treatments that used TMAO in the osmolyte formulations resulted in higher ($p \leq 0.05$) myosin stabilities than the control, especially at day 90 of frozen storage (see Table 2). The synergistic effect of the addition of other osmolytes (Arginine, Taurine, or both) together with TMAO can be detected as higher ($p \leq 0.05$) T_{max}'s values than control were observed at day 90. TMAO is a common osmolyte in high concentration in cold-water fish that functions as a freezing point depressor by increasing their osmotic concentration in blood and tissues (Raymond & DeVries, 1998; MacDonald et al., 2013). It has also been proposed that this osmolyte interacts with forming ice, inhibiting its interaction with the protein (Strambini & Gonnelli, 2008). When comparing Control+ (treatment with the < 1 kDa fraction added) *vs*. Control (washed muscle), the first one also resulted in higher ($p \leq 0.05$) myosin stability (T_{max}) at days 30 and 90 of frozen storage (Table 2). We must mention that a previous analysis of the < 1 kDa fraction showed that TMAO concentration in muscle represents approximately 1.5% (unpublished results).

3.3 Effects of LMMNC on protein surface hydrophobicity

Table 3 shows the protein's surface hydrophobicity changes on samples frozen with the different osmolytes. The addition

Table 1. Effect of osmolytes on Jumbo squid (*Dosidicus gigas*) myofibrillar protein solubility (%) after 90 days of frozen storage (-20°C).

Treatment	Storage days (-20°C)		
	0	30	90
Con	11.7 ± 0.5^{aA}	11.3 ± 0.1^{bCD}	10.7 ± 0.1^{cAB}
Con+	10.4 ± 0.0^{aB}	10.3 ± 0.0^{aB}	10.5 ± 0.3^{aA}
Arg	12.4 ± 0.3^{abC}	12.9 ± 0.3^{aF}	11.5 ± 0.3^{bBC}
Tau	11.2 ± 0.0^{bAD}	10.9 ± 0.2^{bBD}	12.8 ± 0.1^{aD}
TMAO	12.8 ± 0.1^{aC}	12.2 ± 0.1^{bE}	12.9 ± 0.1^{aD}
Arg+Tau	10.6 ± 0.1^{bBE}	9.5 ± 0.3^{cA}	12.7 ± 0.0^{aD}
Arg+TMAO	10.6 ± 0.2^{aBE}	10.9 ± 0.0^{aBC}	11.2 ± 0.2^{aAC}
Tau+TMAO	12.4 ± 0.0^{aC}	11.7 ± 0.2^{aDE}	11.3 ± 0.5^{aAC}
Arg+Tau+TMAO	10.9 ± 0.1^{aBDE}	11.3 ± 0.1^{aCD}	11.2 ± 0.3^{aAC}

Letters A–F in columns and a–c in rows are significantly different ($p \leq 0.05$); Con: control; Con+: positive control; Arg: arginine; Tau: taurine; TMAO: trimethylamine oxide.

Table 2. Jumbo squid (*Dosidicus gigas*) myosin cryostability (T_{max}, °C) conferred by different osmolytes after 90 days of frozen storage (-20°C).

Treatment	Storage days (-20°C)		
	0	30	90
Con	42.6 ± 0.3^{aAB}	42.0 ± 0.1^{aA}	42.0 ± 0.2^{aAB}
Con+	43.6 ± 0.1^{aC}	43.7 ± 0.8^{aBC}	43.3 ± 0.2^{aCE}
Arg	43.4 ± 0.1^{aBC}	42.4 ± 0.1^{bAC}	42.2 ± 0.1^{bAC}
Tau	42.5 ± 0.3^{aAB}	42.0 ± 0.3^{aA}	42.9 ± 0.2^{aBCD}
TMAO	44.3 ± 0.3^{aC}	43.5 ± 0.4^{aAB}	43.4 ± 0.3^{aDE}
Arg+Tau	42.7 ± 0.1^{aAB}	41.9 ± 0.1^{bA}	41.1 ± 0.1^{cA}
Arg+TMAO	44.2 ± 0.9^{abC}	42.7 ± 0.2^{aAC}	45.0 ± 0.6^{aF}
Tau+TMAO	43.5 ± 0.2^{aBC}	43.3 ± 0.6^{aAB}	44.7 ± 0.2^{aF}
Arg+Tau+TMAO	43.4 ± 0.2^{aBC}	45.0 ± 0.4^{bB}	45.5 ± 0.4^{bF}

Letters A–F in columns and a–c in rows are significantly different ($p \leq 0.05$); Con: control; Con+: positive control; Arg: arginine; Tau: taurine; TMAO: trimethylamine oxide.

Table 3. Effect of osmolytes on the surface hydrophobicity of JS (*Dosidicus gigas*) myofibrillar protein after 90 days of frozen storage (-20°C).

Treatment	Storage days (-20°C)		
	0	30	90
Control	0.050 ± 0.006	0.048 ± 0.005	0.058 ± 0.004^{A}
Control+	0.052 ± 0.001	0.052 ± 0.008	0.056 ± 0.004^{AB}
Arg	0.054 ± 0.001	0.050 ± 0.006	0.051 ± 0.008^{AB}
Tau	0.056 ± 0.017	0.051 ± 0.001	0.050 ± 0.001^{AB}
TMAO	0.050 ± 0.001	0.057 ± 0.014	0.048 ± 0.003^{AB}
Arg+Tau	0.060 ± 0.004	0.053 ± 0.013	0.036 ± 0.008^{B}
Arg+TMAO	0.053 ± 0.004	0.051 ± 0.013	0.048 ± 0.005^{AB}
Tau+TMAO	0.056 ± 0.006	0.046 ± 0.000	0.051 ± 0.005^{AB}
Arg+Tau+TMAO	0.054 ± 0.000^{a}	0.044 ± 0.001^{b}	0.045 ± 0.003^{bAB}

Letters A and B in columns and a and b in rows are significantly different ($p \leq 0.05$); Con: control; Con+: positive control; Arg: arginine; Tau: taurine; TMAO: trimethylamine oxide.

of the evaluated LMMNC affected the surface hydrophobicity of the protein on frozen samples, reducing it ($p \leq 0.05$ for Arg+Tau+TMAO) throughout the storage time. Protein conformational changes due to freezing can be deduced from this analysis because this condition can expose the hydrophobic amino acid residues in myosin, leading to a possible protein–protein interaction and resulting in functionality changes. However, it seems that the reduction in protein surface hydrophobicity by LMMNC was due to complex non-covalent binding with the hydrophobic moieties on the myosin fraction (Lv et al., 2022; Yao et al., 2018), leading to less protein–protein interaction, thus improving (or keeping) their solubility, especially at day 90 of frozen storage (as can be observed from Table 1). Other studies have observed that arginine can interact, although weakly, with protein, probably interfering with the hydrophobic surface patches that might form during frozen storage (Stärtzel, 2018), thus avoiding denaturation. Besides, TMAO has been shown to impart excellent freeze/thawing protection to labile proteins, such as enzymes, inhibiting their denaturation during the process (Carpenter & Crowe, 1988; Carpenter et al., 1986).

3.4 Effect of LMMNC on the protein sulfhydryl changes

Freezing/frozen storage can deleteriously affect protein, especially on the sulfhydryl (–SH) functional groups, oxidizing them and forming disulfide bonds (–S–S–). The effects of LMMNC on total –SH content JSMP showed a minimal reduction ($p > 0.05$) throughout the frozen storage time in most treatments (Table 4). However, it seems that the few disulfide bonds formed on samples, besides their reduction in hydrophobicity during the frozen period, promoted the myosin stabilization at the end of the study (day 90) because higher temperatures of denaturation (T_{max}, $p \leq 0.05$) than control (also at day "0") were needed for most treatments (see Table 2), markedly on the ones that TMAO was involved. In this respect, it has been observed that TMAO promotes the formation of disulfide bonds in a model system (Brzezinski & Zundel, 1993). However, based on the results of this study and previous studies at our laboratory, all these changes did not affect the functionality of JSMP.

Table 4. Effect of osmolytes on jumbo squid (*Dosidicus gigas*) myofibrillar protein total sulfhydryl content (μM/mL) after 90 days of frozen storage (-20 °C).

Treatment	Storage days (-20°C)		
	0	30	90
Con	$2.7 \pm 0.1E\text{-}05^{aDE}$	$2.0 \pm 0.1E\text{-}05^{bEFG}$	$2.9 \pm 0.05E\text{-}05^{cB}$
Con+	$3.7 \pm 0.2E\text{-}05^{aA}$	$2.6 \pm 0.1E\text{-}05^{bAC}$	$2.8 \pm 0.1E\text{-}05^{bBC}$
Arg	$3.1 \pm 0.1E\text{-}05^{aBC}$	$2.4 \pm 0.1E\text{-}05^{bBCDE}$	$6.0 \pm 0.3E\text{-}05^{cA}$
Tau	$2.8 \pm 0.1E\text{-}05^{aCDE}$	$2.7 \pm 0.1E\text{-}05^{aAB}$	$2.6 \pm 0.2E\text{-}05^{aBC}$
TMAO	$2.6 \pm 0.1E\text{-}05^{aE}$	$2.2 \pm 0.3E\text{-}05^{aCDF}$	$2.5 \pm 0.0E\text{-}05^{aC}$
Arg+Tau	$3.0 \pm 0.1E\text{-}05^{aCDE}$	$3.0 \pm 0.2E\text{-}05^{aA}$	$2.9 \pm 0.1E\text{-}05^{aB}$
Arg+TMAO	$3.5 \pm 0.2E\text{-}05^{aAB}$	$3.0 \pm 0.1E\text{-}05^{bA}$	$2.9 \pm 0.1E\text{-}05^{bB}$
Tau+TMAO	$3.0 \pm 0.2E\text{-}05^{aCD}$	$3.0 \pm 0.1E\text{-}05^{aA}$	$3.0 \pm 0.1E\text{-}05^{aB}$
Arg+Tau+TMAO	$3.0 \pm 0.1E\text{-}05^{aCDE}$	$2.6 \pm 0.1E\text{-}05^{bADG}$	$2.8 \pm 0.2E\text{-}05^{abBC}$

Letters A–F in columns and a–c in rows are significantly different ($p \leq 0.05$); Con: control; Con+: positive control; Arg: arginine; Tau: taurine; TMAO: trimethylamine oxide.

4 CONCLUSIONS

The influence of different osmolytes present in the < 1 kDa fraction from JS (*Dosidicus gigas*) muscle and their mixtures, especially the ones with Taurine and TMAO, protects its myofibrillar proteins from freezing denaturation/aggregation. These osmolytes are partly why the JS muscle retains its technological functionality even after being frozen. However, evaluating the cryoprotection of other components in the studied fraction, such as carbohydrates and peptides, over the squid muscle proteins is necessary.

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