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Abstract

This study investigated the effects of sucrose and trehalose on pork myofibrillar proteins (MP) subjected to freezing and freeze-drying. Differential scanning calorimetry showed that the addition of sucrose and trehalose increased the glass transition temperature (T'_{g1}) of myofibrillar proteins, while T'_{g1} was not detected in samples without additives. Scanning electron microscopy revealed that freeze-dried samples containing sucrose or trehalose formed uniform and stable porous structures, whereas samples without additives exhibited irregular morphologies. Structural analyses indicated that freezing and freeze-drying of samples without additives significantly increased surface hydrophobicity and reactive sulfhydryl exposure compared with the Raw sample ($p < 0.05$). In contrast, no significant differences were observed between the Raw sample and samples containing sucrose or trehalose ($p > 0.05$). After freeze-drying, carbonyl content analysis showed that MP without additives and the sample containing sucrose exhibited significantly higher carbonyl contents than the Raw sample ($p < 0.05$), whereas no significant difference was observed for the sample containing trehalose compared with the Raw sample ($p > 0.05$). After freeze-drying, samples containing sucrose or trehalose showed significantly higher NaCl-soluble protein solubility than samples without additives ($p < 0.05$). These results indicate that sugar-based excipients influence the structural, oxidative, and functional responses of myofibrillar proteins during freezing and freeze-drying.

Keywords: Freeze-drying, Lyoprotectant, Myofibrillar protein, Sucrose, Trehalose

Introduction

Freeze-drying is a drying method widely used for heat-sensitive materials, in which the sample is first frozen and then dried through sublimation and desorption under pressures below the triple point [1].

34 Compared with other drying methods, freeze-drying induces fewer physicochemical changes, making it
35 suitable for drying protein-rich foods such as meat and beans that are sensitive to heat [2]. In addition, it
36 provides superior retention of nutritional value and flavor and enables long-term storage at ambient
37 temperature after drying [3]. However, even when freeze-drying is applied, protein denaturation can still
38 occur [2]. During freeze-drying, water originally present within the hydration shell of protein is removed,
39 leading to the disruption of hydrogen bonds that link water molecules and proteins [4,5]. The absence of
40 hydrogen bonds that contribute to protein structure increases protein instability, ultimately resulting in
41 denaturation [6]. In addition, freeze-drying generates a highly porous structure, which increases exposure
42 to oxygen and consequently renders the product more susceptible to oxidation [7]. A previous study
43 reported that freeze-drying of pork meat batter altered protein tertiary structure and promoted protein
44 oxidation and aggregation, ultimately leading to reduced gel-forming ability [2].

45 To reduce protein denaturation and oxidation occurring during freeze-drying, lyoprotectants are
46 commonly employed. Among various excipients, polymers, sugars, polyols, and amino acids are
47 representative classes used as lyoprotectants [8]. Because the mechanisms by which excipients protect
48 proteins differ and the regions requiring protection vary among proteins, it is important to select
49 excipients that are appropriate for each specific protein [9]. Among these, trehalose and sucrose are the
50 most widely used lyoprotectants within the sugar class of excipients [10,11]. Trehalose and sucrose are
51 known to reduce structural denaturation by replacing the hydrogen bonds originally formed between
52 proteins and water molecules and by increasing the glass transition temperature (T_g), thereby stabilizing
53 proteins [5,12,13]. Consequently, they have been extensively used as lyoprotectants for protein
54 formulations in the pharmaceutical field [8]. However, most mechanistic insights into lyoprotectant–
55 protein interactions have been derived from studies on purified pharmaceutical proteins, which may be
56 difficult to directly apply to complex food proteins. Although lyoprotectants have been applied in food
57 contexts, these studies have largely focused on surimi-based systems with limited mechanistic
58 investigation [14,15]. Their effect on mammalian muscle proteins, especially under freezing and freeze-
59 drying conditions, remains insufficiently understood.

Therefore, this study aimed to elucidate the protective effects of trehalose and sucrose on pork myofibrillar proteins during freezing and freeze-drying, with particular emphasis on their roles in mitigating structural destabilization, oxidative modification, and functional deterioration.

Materials and Methods

Myofibrillar protein extraction

Ground pork hind legs were obtained from a local butcher shop in Daejeon, South Korea. Myofibrillar proteins (MP) were extracted following the procedure of Lee et al. [16] with slight modifications. All extraction steps were carried out with chilled buffers (4°C, stored overnight) in a 10°C cold room. The ground pork was homogenized with a cold isolation buffer (2 mM MgCl₂, 0.1 M KCl, and 1 mM ethylene glycol tetraacetic acid [EGTA] in 10 mM potassium phosphate, pH 7.0) at a 1:3 (w/v) ratio using a homogenizer (T25 basic, IKA GmbH & Co. KG, Germany) at 13,000 rpm for 30 s, followed by centrifugation at $3,667 \times g$ for 10 min (1580R, LABOGENE Co., Ltd., Lyngby, Denmark), and the pellet was retained. This procedure was repeated twice, with the pellet retained after each centrifugation. The resulting pellet was then resuspended in 100 mM NaCl at the same ratio and processed under identical conditions to obtain the pellet, and this extraction step was also performed twice. The pellet was resuspended with distilled water (1:3, v/v) and centrifuged again at $3,667 \times g$ for 10 min. The final pellet was used for subsequent experiments. The protein concentration of the MP extract, determined by the Kjeldahl method [17], was about 17% (data not shown).

Sample preparation of the myofibrillar protein and the lyoprotectants

Extracted myofibrillar proteins were divided into three groups: a no-exciipient control (No), 10% trehalose (Tre), and 10% sucrose (Suc). Each treatment (20 g) was filled into an aluminum container (w/w, wet sample basis). All samples were frozen at -25°C for 5 h and half of the samples were thawed at 4°C for 12 h. The remaining samples were subsequently freeze-dried using a freeze dryer (MP-05KS, Mareuda, Korea) under < 0.1 mbar. The freeze-drying protocol consisted of sequential drying at shelf

temperatures of -25°C for 24 h (primary drying), followed by 0°C for 24 h and 15°C for 24 h (secondary drying). The freeze-dried samples exhibited a moisture content below 1% (Supplementary Table 1). They were then rehydrated to their original moisture level by adding an equivalent mass of distilled water, calculated from the weight loss between the pre- and post-freeze-drying sample (i.e., water removed during freeze-drying = initial sample weight – freeze-dried sample weight). The calculated amount of distilled water was added to each freeze-dried sample, followed by homogenization using a homogenizer (T25 basic), and the mixture was equilibrated overnight at 4°C to ensure uniform water redistribution prior to analysis. The samples without freezing or drying (Raw), the freeze-thawed (FT), and the freeze-dried–rehydrated (FD) samples were used for subsequent analyses.

Scanning electron microscopy (SEM)

The microstructures of the freeze-dried MP samples were observed following the procedure of Kong et al. [18] with slight modifications. MP samples were observed with a scanning electron microscope (TM4000 Plus II, Hitachi High-Tech, Tokyo, Japan) operated at 15 kV, and images were captured at magnifications of $30\times$ and $100\times$. Each specimen was spread onto a stub using double-sticky carbon tape and subsequently visualized under the microscope. Each specimen was photographed three times.

DSC measurement

Glass transition temperatures of the MP samples were measured using differential scanning calorimetry (DSC 250, TA Instruments, USA). Each sample (20 mg) was loaded into a standard aluminum pan and sealed hermetically with an aluminum lid. Heat flow was monitored across the temperature range, using an empty pan as the reference. Prior to scanning, all samples were annealed at -10°C for 30 min and then heated from -40°C to 10°C at a rate of $10^{\circ}\text{C}/\text{min}$. The glass transition temperature of the maximally freeze-concentrated phase (T'_{g}) was determined from the midpoint of the heat capacity change.

Surface hydrophobicity

The surface hydrophobicity of each MP extract was assessed following the method of Haskard and Li-Chan [19], with minor modifications. Specifically, surface hydrophobicity was quantified through its interaction with 1-(anilino)naphthalene-8-sulfonate (ANS), after adjusting the protein concentration of the samples to 0.1, 0.2, 0.3, 0.4, or 0.5 mg/mL. For ANS binding, 18 μ L of an 8 mM ANS solution prepared in 30% methanol was added to 1.8 mL of each sample and incubated in the dark for 20 min. Fluorescence intensity was then recorded using a plate reader (Varioskan LUX, Thermo Fisher Scientific, USA) with black 96-well plates at excitation and emission wavelengths of 370 and 470 nm, respectively. A trend line was generated from the fluorescence values, and the slope was used to determine the surface hydrophobicity of each sample.

Reactive sulfhydryl content

The reactive sulfhydryl contents of the MP extracts were determined using a modified version of the method reported by Riener et al. [20]. The protein concentration of each sample was adjusted to 1 mg/mL. For the assay, 0.25 mL of the sample was combined with 0.25 mL of 10 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) solution, followed by incubation for 15 min. The absorbance of the resulting mixture was recorded at 412 nm using a plate reader (Varioskan LUX) and corrected using path length correction. Sulfhydryl group content was calculated based on a molar absorptivity of 11,400 $M^{-1}cm^{-1}$.

Carbonyl content

The carbonyl and protein contents were determined according to the procedure of Chen et al. [21], with minor modifications. In brief, carbonyl groups were derivatized with dinitrophenylhydrazine (DNPH), and the absorbance was recorded at 370 nm (Varioskan LUX) to quantify carbonyl levels. Protein content was quantified using the Kjeldahl method [17]. Protein carbonyl concentration was expressed as nmol carbonyl per mg protein, using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

Protein solubility

To assess NaCl soluble protein solubility, 1 g of MP was combined with 20 mL of 0.6 M NaCl buffer and homogenized at 13,000 rpm for 30 s (T25 basic). The homogenate was then centrifuged at 3,000×g for 10 min (1580R), after which the supernatant was collected and filtered through Whatman No. 4 filter paper (Maidstone, UK). The protein concentration of the dissolved (soluble) protein in each sample was determined using a plate reader (Varioskan LUX) with a Bio-Rad protein assay kit (#5000006, Bio-Rad Laboratories, Richmond, CA, USA). A standard curve was prepared using bovine serum albumin. Protein solubility was expressed as the ratio (g/g protein) of NaCl-soluble protein relative to the total protein content. The total protein content of each sample was determined using the Kjeldahl method.

$$\text{NaCl-soluble protein (g/g protein)} = \text{soluble protein (g)} / \text{total protein (g)}.$$

Statistical analysis

Pork MP was produced from hind leg muscles obtained from three independent half-carcasses, which were considered as separate production batches (blocks). For each batch, two independently prepared MP samples were subjected to analysis, serving as technical replicates, and their values were averaged prior to statistical analysis to yield a single observation per batch and treatment combination. Statistical analyses were conducted separately for each processing condition (frozen and freeze-dried) using a randomized complete block design. Treatment (Raw, no excipient, sucrose, and trehalose) was included as a fixed effect and batch (half-carcass) was treated as a random blocking factor. Data were analyzed using a mixed model procedure and results were presented as least-squares means (LS-means) with their standard errors. When a significant effect was detected ($p < 0.05$), multiple comparisons among means were performed using Tukey's multiple-range test. All statistical analyses were carried out with SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Glass transition temperature

Differential scanning calorimetry (DSC) was employed to determine the glass transition temperature (T'_g) of the MP. T'_g is defined as the characteristic temperature at which a partially frozen system shifts from a rubbery state to a glassy state, and the glassy state refers to an amorphous phase with a high viscosity on the order of 10^{12} Pa·s or greater [22]. In this study, T'_g was divided into two parameters: T'_{g1} , defined as the glass transition temperature of the maximally freeze-concentrated phase, and T'_{g2} , defined as the ante-melting temperature at which viscous flow begins in the amorphous region [23].

According to the DSC results, T'_{g1} was not detected in MP without protectants (Fig. 1A). This is presumably because the glass transition of the MP without protectants occurred below the measurable range of the DSC analysis ($< -40^\circ\text{C}$) used in this study. This observation is consistent with Sobral et al. [24], who found that the T'_g of Nile tilapia myofibrillar proteins decreased with increasing moisture, reaching temperatures below 223 K (-50.15°C) at high water activities. Given that the MP used in the present study had a moisture content of more than 80% (data not shown), it is reasonable to infer that its glass transition temperature occurred at temperatures lower than -40°C . However, since a weak or overlapping transition may not have been detected within the examined range, additional experiments are needed for accurate confirmation. In contrast, the addition of sucrose and trehalose resulted in clearly defined T'_{g1} values of -27.08°C and -25.24°C , respectively, with no significant difference ($p > 0.05$) between them (Fig. 1B and C). This result can be attributed to the vitrification effect induced by these sugars. During freezing, sugar-based lyoprotectants immobilize proteins within a rigid glassy matrix, thereby increasing T'_g [8,25]. These findings align with Ohkuma et al. [23], who reported that the addition of sorbitol, glucose, sucrose, and trehalose to carp surimi resulted in a dose-dependent increase in T'_{g1} .

T'_{g2} is the temperature at which ice formation ceases (or ice melting begins), corresponding to a viscosity of approximately 10^7 Pa·s [26,27]. Previous studies have reported that, with the addition of protectants, T'_{g1} increases while T'_{g2} tends to decrease as molecular weight increases, thereby reducing the temperature interval between the two transitions [23,27]. In the present study, a similar trend was observed in the MP upon the addition of sucrose and trehalose, characterized by an increase in T'_{g1} and a decrease in T'_{g2} . The T'_{g2} values for the MP with no protectants, sucrose, and trehalose were -7.34°C ,

191 -7.87°C , and -7.51°C , respectively. However, no significant difference in the T'_{g2} of MP was observed
192 among treatments ($p > 0.05$). This lack of significance might be attributed to the relatively lower addition
193 levels of sucrose and trehalose compared to those used in previous studies.

194 It is common practice to set freeze-drying conditions at a temperature below the collapse temperature.
195 The collapse temperature (T_c) is the temperature above which a freeze-dried product loses its macroscopic
196 structure and collapses during the freeze-drying process [12]. It is generally known that the T_c is
197 approximately $1\text{--}5^{\circ}\text{C}$ higher than the glass transition temperature [30]. Therefore, considering this
198 estimated T_c range, the freezing and primary drying temperature in this experiment was set at -25°C .

200 **Scanning electron microscopy**

201 SEM micrographs provided visual evidence that T'_g -mediated vitrification plays a critical role in
202 governing matrix rigidity during primary drying. High-quality freeze-dried products should exhibit a
203 uniform cake structure without collapse [28]. MP samples without protectants exhibited irregular and
204 discontinuous pore structures (Fig. 2A and B). In general, freeze-drying produces a porous matrix and
205 forms a characteristic cake structure. However, if drying proceeds at temperatures above the T_c , structural
206 collapse may occur within the cake, potentially resulting in a non-porous structure [8,29]. When freeze-
207 drying is conducted above the T_c , the product matrix becomes mobile, which can lead to cake collapse
208 [29]. MP without protectants exhibited no T'_{g1} between -40°C and 10°C (Fig. 1A), indicating that the
209 primary drying temperature exceeded the T_c , which was consistent with the observed structural instability.
210 In contrast, MP samples containing sucrose or trehalose displayed a uniform and well-defined cake
211 morphology (Fig. 2C-F). Sugar-based lyoprotectants such as sucrose and trehalose promote the formation
212 of a highly viscous state, thereby supporting the development of a stable cake structure [8]. According to
213 our DSC measurements, the T'_{g1} values of MP samples containing sucrose and trehalose were -27.08°C
214 and -25.24°C , respectively (Fig. 1B and C). Because freeze-drying in this study was performed at -25°C ,
215 the process proceeded near the T_c for these formulations. Collectively, these results demonstrate that the
216 addition of lyoprotectants increased T'_g , enabling the matrix to avoid collapse throughout the freeze-

drying process and resulting in the formation of uniform and stable cake structures. Similar trends have been reported in previous studies. Zheng [28] showed that freeze-dried bacteriophages formulated only with PBS buffer exhibited pronounced cake collapse, whereas formulations containing sucrose or trehalose produced well-preserved and stable cake structures. Li et al. [31] reported that freeze-drying mRNA-LNP formulations containing 8.8% sucrose, 2% trehalose, and 0.04% mannitol yielded a rigid, ginger-root-like morphology with high porosity as observed by SEM. Similarly, Zhao et al. [32] demonstrated that duck hepatitis virus vaccines freeze-dried with 10% sucrose, 1.2% pullulan, 0.5% PVP, and 1% arginine exhibited a porous, honeycomb-like structure under SEM analysis.

MP denaturation

MP denaturation during FT and FD was assessed by monitoring surface hydrophobicity and reactive sulfhydryl content as indicators of tertiary structural changes. Following FT, the No (FT without protectants) exhibited significantly higher surface hydrophobicity than the Raw (non-FT) sample ($p < 0.05$, Fig. 3A). During freezing, MP undergoes cold denaturation as hydrophobic stabilization is weakened due to the reduced entropic driving force at low temperature. Consequently, hydrophobic residues that are normally buried within the protein interior become exposed, leading to increased surface hydrophobicity. Zhang and Ertbjerg [33] reported that freezing pork loin at -18°C and -80°C for 24 h, followed by thawing, increased the surface hydrophobicity of isolated MP at both temperatures. In contrast, Suc and Tre treatments showed no significant difference in surface hydrophobicity compared with the Raw sample ($p > 0.05$, Fig. 3A). The addition of sucrose and trehalose increased the T'_{g1} , enabling the formation of a glassy state even when freezing of all samples was conducted at the same temperature (Fig. 1B and C). In a glassy state, the markedly increased viscosity restricts protein conformational relaxation and limits interconversions among conformational substates, thereby enhancing structural stability during freezing [5,34]. In addition, the incorporation of sucrose and trehalose further stabilizes MP during freezing via preferential exclusion, which promotes preferential hydration of MP and increases the free energy barrier for unfolding [5]. Preferential exclusion reflects the thermodynamically

unfavorable presence of these solutes near the protein surface, resulting in preferential hydration and a higher local concentration of water molecules surrounding the protein relative to the bulk solution. As a result, exposure of hydrophobic domains is disfavored, and protein unfolding requires expansion of the protein–solvent interface accompanied by displacement of excluded solutes [5,8,35,36]. Therefore, increased T'_g and preferential exclusion induced by sucrose and trehalose synergistically suppress unfolding of MP during the FT process. Consistent with these findings, Sompongse et al. [37] reported that actomyosin extracted from *Cyprinus carpio* and supplemented with 20% sucrose exhibited significantly lower surface hydrophobicity during ice storage compared with untreated samples ($p < 0.05$).

After the FD process, the No exhibited significantly higher surface hydrophobicity than the Raw sample ($p < 0.05$, Fig. 3B). During the FD process, the removal of water that stabilizes the folded protein structure induces denaturation, leading to alterations in both secondary and tertiary structures. Dehydration disrupts intramolecular interactions and hydration shells, resulting in conformational destabilization and exposure of hydrophobic residues that are normally buried within the protein core [38,39]. A similar result was reported by Han et al. [2], who observed higher surface hydrophobicity in myosin extracted from freeze-dried meat batter than from frozen–thawed meat batter. In contrast, no significant differences in surface hydrophobicity were observed between freeze-dried samples (Suc and Tre) and the Raw sample ($p > 0.05$, Fig. 3B). These results suggest that sucrose and trehalose functioned as lyoprotectants by maintaining protein conformation during the FD process. In the removal of water, these disaccharides can substitute for hydration by forming hydrogen bonds with polar moieties on the protein surface, thereby stabilizing the native structure [13]. Their abundant hydroxyl (–OH) groups facilitate extensive hydrogen bonding, which helps preserve intramolecular interactions and prevent structural collapse during dehydration [5,40]. Accordingly, the addition of sucrose and trehalose is considered to suppress MP denaturation during the FD process.

Analysis of reactive sulfhydryl groups showed that, during the FT process, no significant differences were observed among treatments compared with the Raw sample ($p > 0.05$, Fig. 4A). This is likely because the 5 h freezing period was relatively short and insufficient to induce disulfide bond disruption or

thiol exposure. Similarly, Ali et al. [41] reported no significant changes in sulfhydryl content after a single freeze–thaw cycle ($p > 0.05$), in which chicken breast was frozen at -20°C and thawed at 4°C .

After the FD, the No exhibited significantly higher reactive sulfhydryl content than the Raw sample ($p < 0.05$, Fig. 4B). This increase can be attributed to partial denaturation of MP during the FD process [42]. Upon denaturation, thiol groups that are normally buried within the protein interior become exposed, resulting in increased reactive sulfhydryl content [43]. In contrast, no significant differences were observed between Suc and Tre treatments and the Raw sample ($p > 0.05$, Fig. 4B). These results suggest that sucrose and trehalose functioned as lyoprotectants by suppressing MP denaturation and limiting thiol exposure during the FD. A comprehensive evaluation of tertiary structural parameters further confirmed that FD induced MP denaturation, as evidenced by increases in both surface hydrophobicity and reactive sulfhydryl content. However, when FD was performed in the presence of sucrose or trehalose, structural integrity was preserved at levels comparable to the non-FD Raw sample. Collectively, these findings demonstrate that sugar-based lyoprotectants effectively mitigate structural alterations of MP during the FD process.

Protein oxidation

To evaluate protein oxidation induced by FT and FD, carbonyl content was determined as a marker of oxidative modification. All FT-treated samples (No, Suc, and Tre) exhibited significantly higher carbonyl content than the Raw sample ($p < 0.05$, Fig. 5A). Protein oxidation leads to various chemical modifications, among which carbonylation is one of the most representative. Carbonyl groups are primarily generated through oxidative reactions in side chains of amino acids such as lysine, proline, threonine, and arginine, which are abundant in MP [3]. During freezing, protein oxidation can be promoted by multiple factors, including pH shifts due to freeze concentration, physical disruption caused by ice crystal formation, and adsorption of proteins at ice-water interfaces. Du et al. [44] reported that a single freeze–thaw cycle significantly increased the carbonyl content of MP extracted from mirror carp. Among the FT-treated samples, the Tre treatment exhibited significantly lower carbonyl content than the

No and Suc treatments ($p < 0.05$, Fig. 5A), indicating enhanced protection against oxidation. Trehalose is often reported to provide greater cryoprotective efficacy than sucrose, partly due to its higher hydration capacity and ability to more effectively modulate water structure in a freeze-concentrated system [45]. This enables trehalose to better inhibit ice crystal growth and reduce ice-induced physical damage. In addition, trehalose has been reported to exhibit approximately twofold higher ice growth inhibition activity than sucrose [46]. Therefore, reduced ice crystal formation and growth by trehalose in FT-treated MP mitigated structural damage and limited oxidative reactions, resulting in lower protein oxidation ($p < 0.05$).

After the FD, the No treatment showed significantly higher carbonyl content than the Raw sample, indicating that FD induced substantial protein oxidation in MP ($p < 0.05$, Fig. 5B). During the FD, sublimation of ice crystals generates a porous structure and increases oxygen exposure, which facilitates oxidative reactions [7]. Therefore, protein oxidation generally occurs during the FD. Consistent with this, Lee et al. [16] reported that pork subjected to FD exhibited significantly higher carbonyl content than pork subjected only to FT treatment ($p < 0.05$), and Han et al. [2] observed similar increases in myosin extracted from FD pork meat batter ($p < 0.05$). The Suc treatment showed no significant difference in carbonyl content compared to the No treatment ($p > 0.05$). This result was consistent with the result of the carbonyl content in FT-treated samples, suggesting limited protective efficacy of sucrose against MP oxidation under these conditions. In contrast, the Tre treatment exhibited significantly lower carbonyl content than the No treatment and did not differ significantly from the Raw sample. This enhanced stability of MP in the Tre treatment can be attributed to differences in physicochemical state during the FD. Specifically, the No sample was subjected to temperatures above T'_{g1} whereas the Tre-treated sample remained near T'_{g1} (Fig. 1), favoring a glassy state with restricted molecular mobility. In addition, as described above, trehalose more effectively inhibits ice crystal formation and growth, thereby reducing structural damage during freezing and subsequent drying. Therefore, reduced structural denaturation and unfolding in the Tre treatment decreased the susceptibility of MP to oxidative modification ($p < 0.05$).

Protein solubility

To evaluate protein functionality after FT or FD, protein solubility was measured. The results showed that solubility decreased significantly in all FT-treated samples, regardless of protectant incorporation, compared to the Raw treatment ($p < 0.05$, Fig. 6A). This reduction is attributable to increased protein oxidation observed after FT ($p < 0.05$, Fig. 5A). Oxidative modification, such as carbonylation, leads to the loss of protonatable amino groups and alters the charge distribution and overall electrostatic properties of MP [47]. These changes weaken electrostatic repulsion and promote protein-protein interactions over protein-water interactions, resulting in aggregation and reduced solubility [47]. Consistently, Zhang et al. [48] reported that pork MP treated with hydrogen peroxide (H_2O_2) decreased solubility in parallel with increased carbonyl content ($p < 0.05$). Utrera and Estévez [49] demonstrated similar reductions of solubility in pork MP following oxidation induced by iron, myoglobin, or copper ($p < 0.05$). In addition to oxidation, conformational denaturation, particularly protein unfolding, also affects solubility. Increased surface hydrophobicity promotes protein-protein aggregation, further decreasing solubility [16]. Although Suc and Tre treatments did not show a significant difference in surface hydrophobicity compared with the Raw treatment, their solubility remained lower than that of the Raw treatment ($p < 0.05$, Fig. 6A). This suggested that MP solubility is governed by combined effects of oxidative modification and structural denaturation.

After FD, all FD-treated samples exhibited significantly lower solubility than the Raw sample ($p < 0.05$, Fig. 6B), which could be attributed in part to oxidative modifications occurring during the FD (Fig. 5B). However, the Suc and Tre treatments showed significantly higher solubility than the No treatment ($p < 0.05$, Fig. 6B). This improvement can be explained by the reduced structural denaturation in Suc and Tre treatments, as evidenced by their lower surface hydrophobicity and reactive sulfhydryl content compared with the No treatment ($p < 0.05$, Fig. 3B and 4B). In addition, the No treatment exhibited the highest level of protein carbonylation, whereas the Tre treatment showed significantly lower carbonyl content than the No treatment ($p < 0.05$, Fig. 5B), indicating reduced oxidative modifications. Therefore, the combined effects of enhanced structural stability and limited oxidative modification in the presence of sucrose and

347 trehalose contributed to mitigating the loss of MP solubility during the FD. In meat processing, the
348 functionality of MP depends on the extraction of salt-soluble proteins, which are essential for gelation,
349 emulsification, and water-holding capacity. Accordingly, the use of sucrose and trehalose as protectants
350 may hold potential for improving the processing suitability of FD meat by preserving protein solubility.

352 **Conclusion**

353 This study evaluated the suitability of sucrose and trehalose as lyoprotectants for pork MP by
354 examining structural stability, oxidative susceptibility, and protein solubility during FT and FD processes.
355 Both FT and FD induced structural denaturation and oxidative modification in MP. However, the
356 incorporation of sucrose and trehalose inhibited these changes and consequently mitigated the loss of MP
357 solubility during the FD. The addition of sucrose and trehalose increased T'_{g1} , enabling FD to occur near
358 the T_c and thereby promoting the formation of a stable glassy state. Under these conditions, molecular
359 mobility is restricted, limiting cold denaturation and oxidation reactions. Furthermore, trehalose provided
360 superior protection against freeze-induced protein oxidation compared with sucrose during the freezing
361 stage, whereas both disaccharides showed comparable efficacy in preserving protein structure and
362 solubility following FD. Nevertheless, these lyoprotectants did not fully preserve MP during the FD
363 compared to the raw sample with no FD. Therefore, further studies exploring a broader range of
364 concentrations for commercial use, combinations of protectants, and alternative classes of lyoprotectants
365 are warranted to better understand formulation-dependent effects and to optimize freeze-drying strategies
366 for meat protein systems. For a more detailed discussion supporting this conclusion, it would be necessary
367 to additionally compare the effects of simply adding sugars to MP without any other treatment, as well as
368 to compare freezing and freeze-drying directly.

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Figure Captions

Figure 1. Heat flow (W/g) of pork myofibrillar proteins (MP) with different protectants determined by DSC analysis.

The values are the LS-means.

T'_{g1}, glass transition temperature of the maximally freeze-concentrated phase; T'_{g2}, ante-melting temperature at which viscous flow begins in the amorphous region

Figure 2. Scanning electron microscopy (SEM) images of freeze-dried pork myofibrillar proteins with different protectants.

MP, myofibrillar protein.

Figure 3. S₀-ANS surface hydrophobicity of pork myofibrillar proteins subjected to frozen–thawed (FT, A) or freeze-dried and rehydrated (FD, B) treatments with different protectants.

Error bars represent the standard error of LS-means (n = 3).

^{A,B}Different uppercase letters indicate significant differences in the means between presence or type of additive ($p < 0.05$).

Raw, myofibrillar protein without freezing–thawing or freeze-drying treatment; No, myofibrillar protein subjected to freezing–thawing or freeze-drying without any excipient; Suc, myofibrillar protein subjected to freezing–thawing or freeze-drying with sucrose; Tre, myofibrillar protein subjected to freezing–thawing or freeze-drying with trehalose.

Figure 4. Reactive sulfhydryl groups (nmol/mg) of pork myofibrillar proteins subjected to frozen–thawed (FT, A) or freeze-dried and rehydrated (FD, B) treatments with different protectants.

Error bars represent the standard error of LS-means (n = 3).

^{A,B}Different uppercase letters indicate significant differences in the means between presence or type of additive ($p < 0.05$).

Raw, myofibrillar protein without freezing–thawing or freeze-drying treatment; No, myofibrillar protein subjected to freezing–thawing or freeze-drying without any excipient; Suc, myofibrillar protein subjected to freezing–thawing or freeze-drying with sucrose; Tre, myofibrillar protein subjected to freezing–thawing or freeze-drying with trehalose.

542

543 **Figure 5. Protein carbonyl content (nmol/mg) of pork myofibrillar proteins subjected to**
544 **frozen–thawed (FT, A) or freeze-dried and rehydrated (FD, B) treatments with different**
545 **protectants.**

546 Error bars represent the standard error of LS-means ($n = 3$).

547 ^{A-C}Different uppercase letters indicate significant differences in the means between presence
548 or type of additive ($p < 0.05$).

549 Raw, myofibrillar protein without freezing–thawing or freeze-drying treatment; No,
550 myofibrillar protein subjected to freezing–thawing or freeze-drying without any excipient; Suc,
551 myofibrillar protein subjected to freezing–thawing or freeze-drying with sucrose; Tre,
552 myofibrillar protein subjected to freezing–thawing or freeze-drying with trehalose.
553

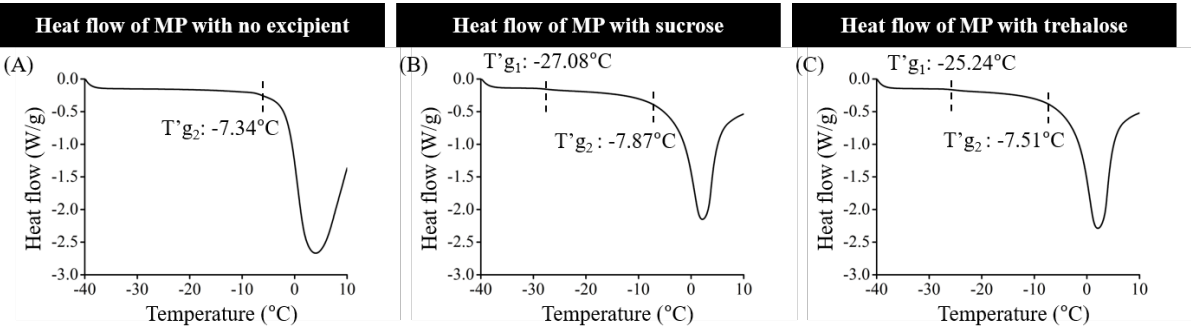
554 **Figure 6. Protein solubility (g/g protein) of pork myofibrillar proteins subjected to**
555 **frozen–thawed (FT, A) or freeze-dried and rehydrated (FD, B) treatments with different**
556 **protectants.**

557 Error bars represent the standard error of LS-means ($n = 3$).

558 ^{A-C}Different uppercase letters indicate significant differences in the means between presence
559 or type of additive ($p < 0.05$).

560 Raw, myofibrillar protein without freezing–thawing or freeze-drying treatment; No,
561 myofibrillar protein subjected to freezing–thawing or freeze-drying without any excipient; Suc,
562 myofibrillar protein subjected to freezing–thawing or freeze-drying with sucrose; Tre,
563 myofibrillar protein subjected to freezing–thawing or freeze-drying with trehalose.
564

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566

567 **Figure 1**

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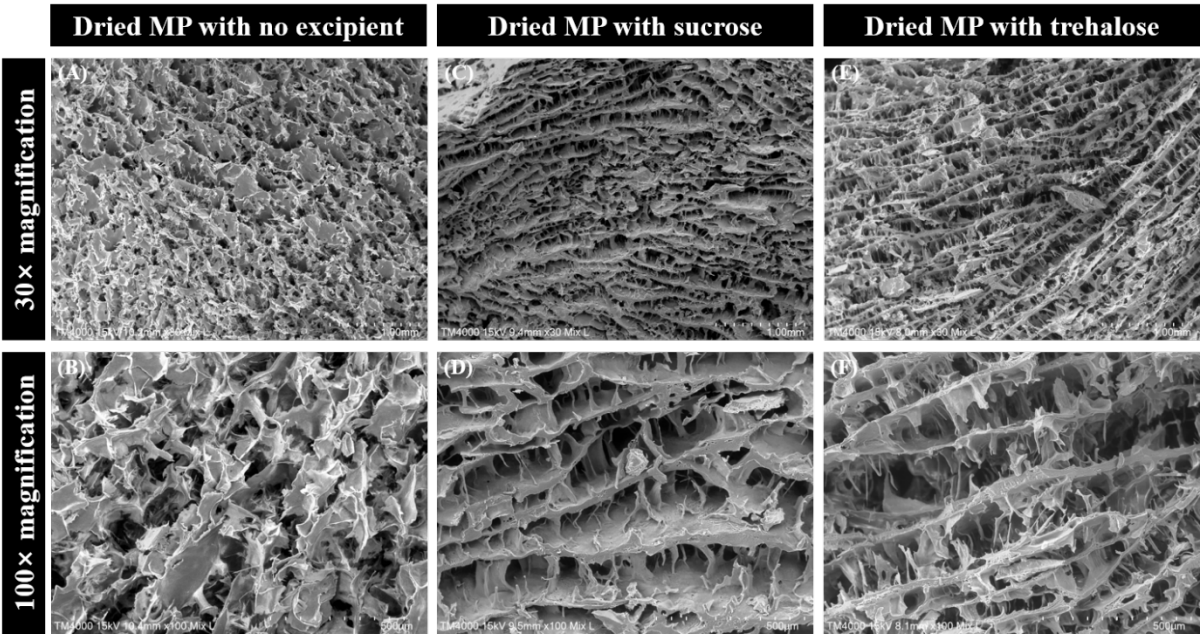
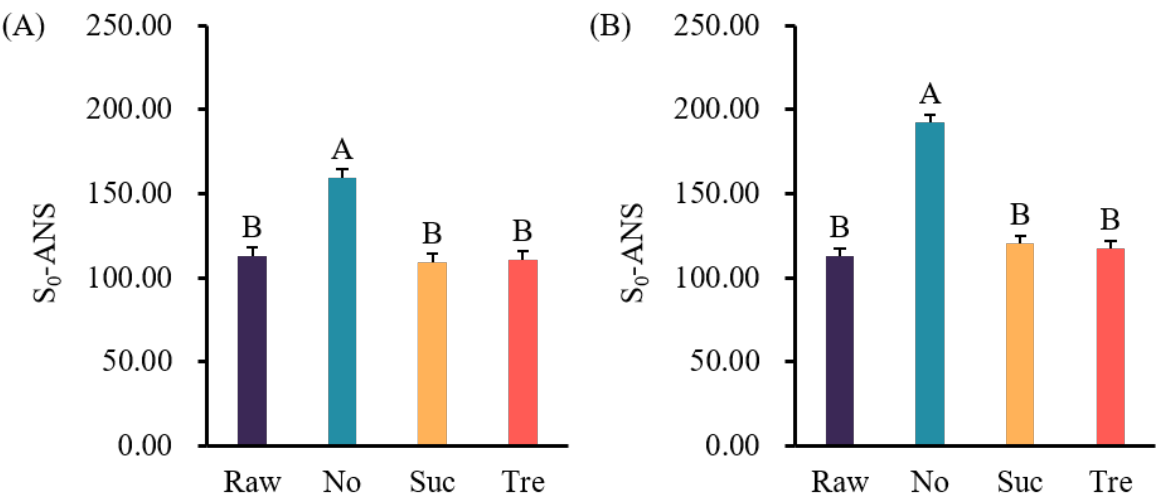


Figure 2

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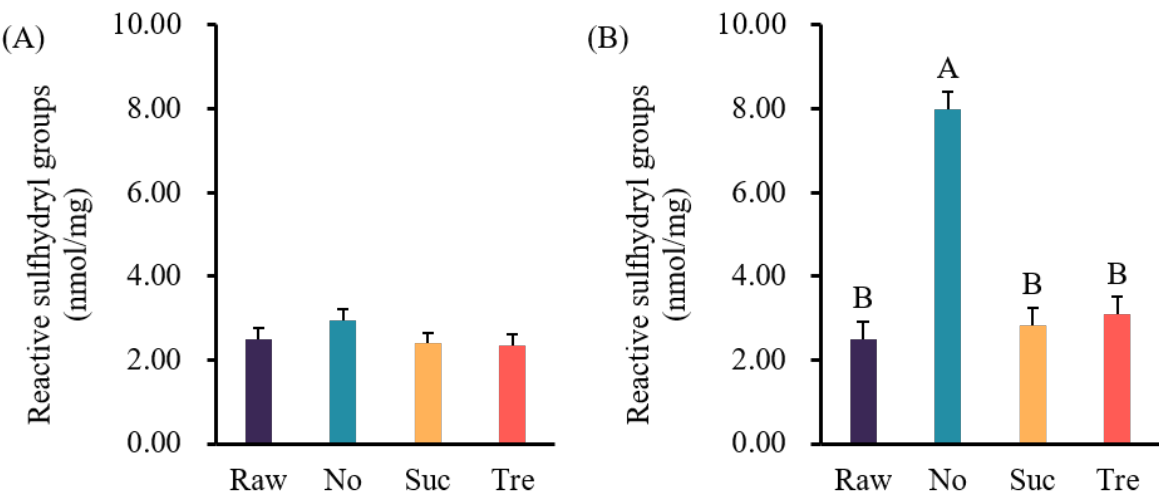


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576 **Figure 3**

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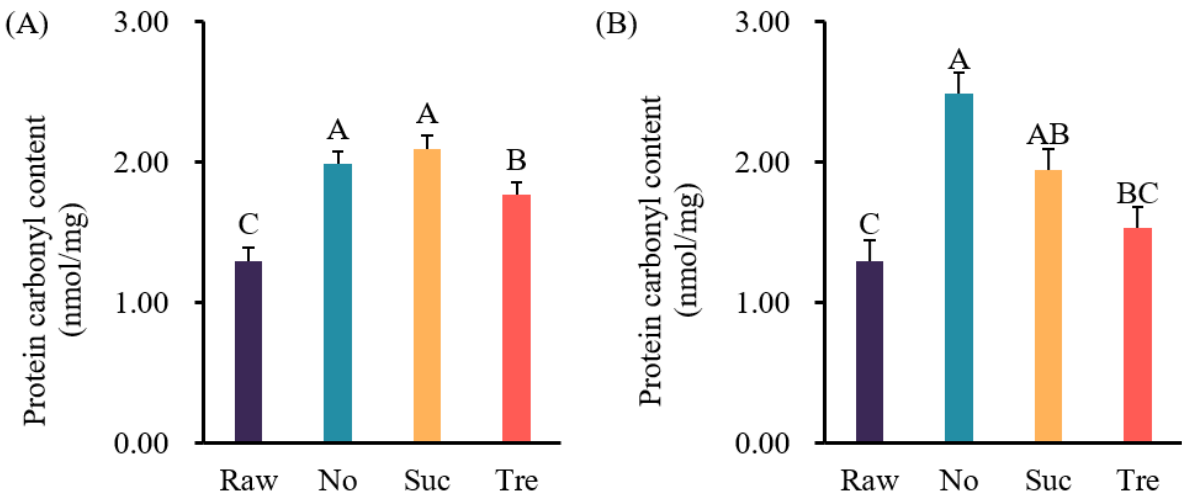


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580 **Figure 4**

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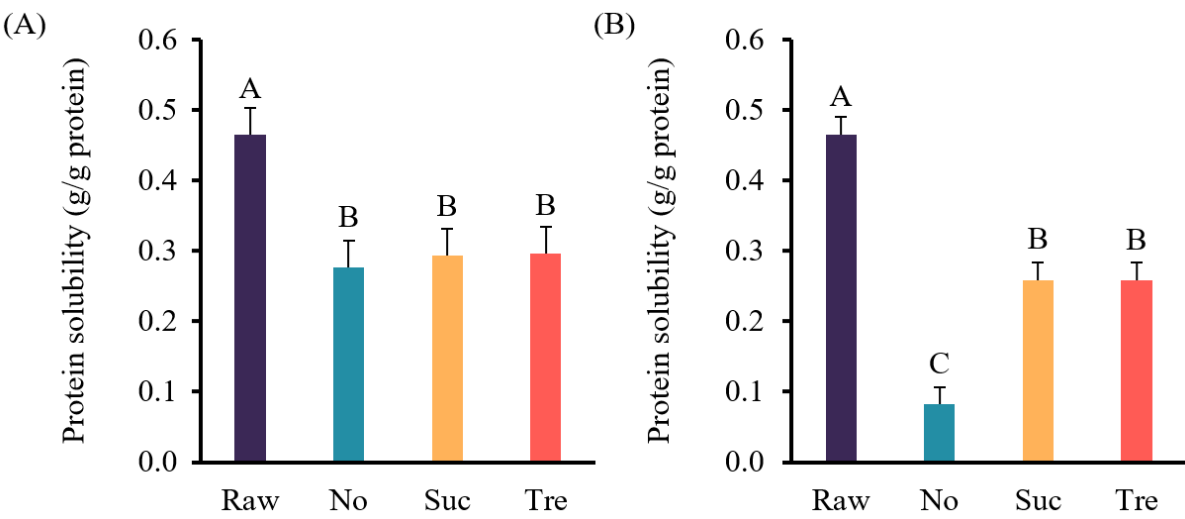


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584 **Figure 5**

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588 **Figure 6**

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Supplementary Table 1. Residual moisture content of freeze-dried myofibrillar protein

Treatment ²	No	Suc	Tre	SEM ¹
Residual moisture content	0.99	0.82	0.85	0.073

(MP) samples.

¹Standard error of the least-squares means (n = 3).

²No, myofibrillar protein subjected to freezing–thawing or freeze-drying without any excipient; Suc, myofibrillar protein subjected to freezing–thawing or freeze-drying with sucrose; Tre, myofibrillar protein subjected to freezing–thawing or freeze-drying with trehalose.