Usage of nanocrystalline cellulose as a novel cryoprotective substance for the *Nemipterus virgatus* surimi during frozen storage

Zhengyi Li\(^a,1\), Qi Wang\(^b,3,1\), Shuting Li\(^a\), Yu Chang\(^a\), Xuan Zheng\(^a\), Hui Cao\(^c,e,\ast\), Yafeng Zheng\(^b,2\)

\(^a\) College of Food Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China  
\(^b\) Institute of Agricultural Engineering, Fujian Academy of Agriculture Sciences, Fuzhou, Fujian 350003, China  
\(^c\) Universidade de Vigo, Nutrition and Bromatology Group, Department of Analytical and Food Chemistry, Faculty of Sciences, 32004 Ourense, Spain  
\(^d\) Fujian Key Laboratory of Agricultural Product (Food) Processing, Fuzhou 350003, China  
\(^e\) College of Food Science and Technology, Guangdong Ocean University, Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety, Zhanjiang 524088, China

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**ABSTRACT**

Using a novel natural cryoprotectant to maintain better quality of frozen aquatic products is attracting increasing interests in recent years. This study investigated the cryoprotective effects of bamboo shoot nanocrystalline cellulose (NCC) on *Nemipterus virgatus* surimi during 60 days of frozen storage. Compared with surimi without any cryoprotectant, NCC addition significantly retard the quick decrease of salt soluble protein content, Ca\(^{2+}\)-ATPase activity and sulfhydryl content of myofibrillar protein. SDS-PAGE results suggested that NCC could protect the structural integrity of myofibrillar protein, which was confirmed by the enhanced stability of \(\alpha\)-helix content of protein. It was also observed that surimi gel incorporated with NCC showed improved gel strength and a more compact microstructure with dense surfaces. This study demonstrated that NCC could potentially serve as a novel natural cryoprotectant for the surimi.

**Introduction**

Seafood, which contains abundant nutritional compounds, is one of the important high-quality protein sources for humans. To improve the utilization of seafood resource, large amounts of inexpensive fish meat are minced to produce surimi, which is mainly comprised of concentrated myofibrillar proteins (Tang et al., 2019). Moreover, due to its unique gel-forming properties, surimi can be further processed with additives and molded into various more expensive products such as imitation crab meat, fish ball, fish cake and tempura (Yi, Ye, Li, Wang & Li, 2020).

Frozen storage is the main method to preserve the quality of surimi. However, some undesirable changes such as protein denaturation, oxidation and growth of ice crystals are still taking place during frozen storage and causing irreversible damage to surimi quality (Nikoo, Benjakul, & Rahmanifar, 2016; Wu, Pan, & Wang, 2014). To delay these negative changes, some commercial cryoprotectants, such as sucrose and sorbitol, have been commonly used to ensure maximum functionality of frozen surimi. However, high sweetness and caloric content of sucrose and sorbitol are not suitable for a healthy diet and some specific consumers suffering from diabetes. To overcome these defects, a comprehensive investigation for alternative cryoprotectants has been carried out in recent years. Some low-calorie and sweetness cryoprotectants, such as polydextrose (Nopianti, Huda, Noryati, Fazilah, & Easa, 2012), resistant starch (Yang and Wang, & Ye, 2014), chitosan (Dey & Dora, 2011), oligosaccharides (Zhang, Yang, Tang, Hao, Zhang, & Deng, 2017) and protein hydrolysates (Chen et al., 2022; Zhang, Li, Hong, & Luo, 2020), have been investigated for their cryoprotective roles in surimi or seafoods during frozen storage.

Rapid development of nanotechnology promoted the investigation and application of nano-grade food compounds in the development of healthier and safer foods (Jagtiani, 2022; Mu et al., 2019). Compared to large particles, nanoparticles with same composition could exhibit greater chemical and biological activities. Nanocrystalline cellulose...
(NCC), also named as cellulose nanocrystalline, is a nano-sized rod-shaped crystal prepared from cellulose, the most abundant natural resource (Yu, Yang, Huang, Cao, Yang, & Liu, 2012). NCC is commonly derived from various botanical processing byproducts. With the advantages of high crystallinity, small aspect ratio and large specific surface area, and containing a large number of negatively charged hydroxyl groups on its surface, NCC has been widely used as a stabilizing and functional food ingredient such as dietary fiber (Gómez et al., 2016). Adding nanosized dietary fiber to surimi improves not only its nutritional composition by increasing the dietary fiber content, but also its gelation properties possibly owing to the interactions among fiber, protein and water molecules in the gel matrix (Yin et al., 2019). However, there are few studies that discuss the possible cryoprotective roles of NCC in surimi during frozen storage and the subsequent effect on surimi gel quality.

Golden threadfin bream (Nemipterus virgatus) is an important marine fish species in Southeast Asian and is commonly used for surimi production (Zhu, Lanier, & Farkas, 2015). This study investigated the cryoprotective effects of NCC on the Nemipterus virgatus surimi during 60 days of frozen storage, in order to explore the usage of NCC as a novel and heathier surimi cryoprotectant.

Materials and methods

Materials and reagents

Fresh golden threadfin bream (Nemipterus virgatus) were obtained from the local supermarket in Fuzhou, China. Additionally, bovine serum albumin (BSA), tris and sodium dodecyl sulfate (SDS) were purchased from Solarbio Life Science Co., Ltd., Beijing, China. All other chemicals used were of analytical reagent grade.

Preparation of NCC and surimi samples

NCC was extracted from defatted bamboo shoot powder, provided by Mingliang Food Co., Ltd., Jianou, China, according to the reported method with minor modifications (Yu et al., 2012). The bamboo shoot powder (10.0 g) was treated by 3 wt% NaOH aqueous solution (200 mL) at 50 °C for 2 h and then washed with water until the residue was free from acid. The treated samples were further hydrolyzed by sulfuric acid (50 wt%, 200 mL) at 50 °C with mechanical stirrer 1,200 rpm for 30 min. The acidolysis reaction was stopped by washing four times with distilled water and centrifugation (8,000 rpm, 10 min). The suspension was then filtered with distilled water in a dialysis bag for 2 days until the pH reached neutral. Then, the resultant suspension was sonicated for 30 min and freeze-dried to obtain the rod-like NCC with length of 123–230 nm and diameter around 13 nm.

Fresh golden threadfin bream were packed in an ice box and immediately transported to the laboratory. After the fishes were washed thoroughly with distilled water, the head, scale and viscera were removed, and the fish meat was collected manually. The meat was cut into fillets, minced and washed three times (twice with distilled water and once with 0.15% NaCl solution, stirred for 5 min each and left to stand for 5 min). After dehydration with gauze, the surimi was obtained. The surimi samples were prepared by mixing 2%, 4%, 6% and 8% (w/w) of NCC, respectively. The common commercial agent (sucrose/sorbitol, 1:1, 8% w/w) was added to surimi to prepare a positive control (PC), while surimi without adding any cryoprotective substance was used as a negative control (NC). The samples were separately packed into polyethylene casings and stored at −18 °C for 60 days. The indexes were measured every 12 days.

Extraction of myofibrillar protein

Myofibrillar protein was extracted at a controlled condition (−4 °C) according to a modified method (Lin, Hong, Zhang, Zhang, & Luo, 2019), and the centrifuge speed was set at 12,000 rpm. Briefly, 2.0 g of frozen surimi was homogenized with 15 mL distilled water (4 °C) and centrifuged for 15 min. The precipitate was collected and homogenized with 15 mL ice-cold low salt buffer solution (0.1 M NaCl, 20 mM Tris–maleate, pH 7.2). The precipitates were separated and homogenized with 30 mL ice-cold high salt buffer solution (0.6 M NaCl, 20 mM Tris–maleate, pH 7.2), incubated at 4 °C for 1 h and centrifuged for 10 min. The supernatant was collected and diluted with 4 volumes of cooled distilled water and centrifuged for 10 min to precipitate the myofibrillar protein. The sediment was dissolved with 0.6 M NaCl (pH 7.0) and centrifuged for 5 min to remove the insoluble substances.

Determination of salt soluble protein content

Salt soluble protein was extracted from frozen surimi (2.0 g) using the same extraction method of myofibrillar protein with some modifications at final two steps. After the supernatant was diluted with 4 volumes of cooled distilled water, the salt soluble protein was fully solubilized after 1 h incubation at 4 °C and then centrifuged for 10 min. The relative salt soluble protein content was determined by the Biuret method (Gornall, Bardawill, & David, 1949) and calculated by the following equation:

\[ \text{Relative salt soluble protein content (\%)} = \frac{C_t}{C_0} \times 100 \]

where \( C_t \) is the protein concentration of the supernatant after frozen storage, \( C_0 \) is the protein concentration of the supernatant from fresh surimi sample.

Determination of Ca\(^{2+}\)-ATPase activity

The Ca\(^{2+}\)-ATPase activity was determined according to the method described by Liu et al. (Liu, Fang, Luo, Ding, & Liu, 2019) with some modifications. The Ca\(^{2+}\)-ATPase activity of proteins was determined at 25 °C in a reaction solution containing 5 mM CaCl\(_2\), 1 mM adenosine triphosphate (ATP), 0.5 M NaCl, 25 mM Tris–maleate (pH 7.0) and 6.0 mg/mL protein sample for 10 min. HClO\(_4\) was added at a final concentration of 5% to terminate the reaction. The solution was centrifuged at 6000 rpm for 10 min to clarify the supernatant. Then the inorganic phosphate liberated in the solution was assayed by complexing with ammonium molybdate and measuring the absorbance at 820 nm (Saunders et al., 1997). Ca\(^{2+}\)-ATPase activity was expressed as μmol of inorganic phosphate (Pi) released per mg of myofibrillar protein per reaction time (μmol/mg/min), and calculated by the following equation:

\[ \text{Relative Ca}^{2+} - \text{ATPase activity (\%)} = \frac{D_t}{D_0} \times 100 \]

where \( D_t \) is Ca\(^{2+}\)-ATPase activity during frozen storage, \( D_0 \) is Ca\(^{2+}\)-ATPase activity at the beginning of storage.

Determination of sulfhydryl content

The sulfhydryl content of myofibrillar protein was determined following a previously reported method (Lin et al., 2019). After myofibrillar protein was diluted to 2 mg/mL, the sulfhydryl content was determined in a solution containing 0.25 mM protein sample, 50 μL 10 mM 5,5’-Dithiobis-(2-nitrobenzoic acid) (DTNB), 2.5 mL of 8 M urea, 10 mM ethylene-diaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS) and 0.2 M Tris-HCl (pH 7.1) at 40 °C for 15 min. The mixture was cooled to ambient temperature and the absorbance of the supernatant was measured at 412 nm. The sulfhydryl content was calculated using a molar extinction coefficient of 13,600 mol/cm, and was expressed as mol/10\(^5\) g protein.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was carried out using the described method
(Zhang, Fang, Hao, & Zhang, 2018) on 10 % resolving gel with 5 % stacking gel. Samples were prepared by mixing diluted protein samples (10 mg/mL) with loading buffer in a ratio 3:1 and heating the mixture to 100 °C for 5 min to denature the protein samples. A loading volume of 10 μL was added to each line. After electrophoresis, gels were stained with 0.1 % (w/v) Coomassie Brilliant Blue in a solution containing 80 % methanol and 20 % acetic acid and destained in a solution containing 50 % methanol and 10 % acetic acid. An SDS-PAGE broad-range molecular weight standard (11–245 kDa, SolelyBio, Beijing, China) was used.

### Fourier transform infrared spectra (FT-IR) analysis

Fourier transform infrared spectra (FT-IR) analysis of myofibrillar protein was based on a described method with minor modifications (Zhao, Han, Wen, Xia, Chen, & Kong, 2020). The FT-IR measurements from 4000 to 400 cm$^{-1}$ were carried out on an FT-IR instrument (VERTEX 70 Bruker, Germany) with 32 scans at a resolution of 4 cm$^{-1}$. The myofibrillar protein was mixed with potassium bromide (KBr) in a mass ratio of 1:100 and milled evenly. Quantitative analysis of secondary structures of myofibrillar protein was performed using the Peak Fit v 4.12 software. The relative areas of individual assigned bands in the amide I region (1600–1700 cm$^{-1}$) were used for calculating the secondary structural components, including α-helix, β-sheet, β-turn, and random coil.

### Preparation of surimi gel

Surimi gel was prepared using the reported methodology (Lin et al., 2019) with some slight changes. Frozen surimi was thawed and blended thoroughly with 2.5 % (w/w) NaCl solution. Afterward, the mixture was stuffed and sealed with vinylidene chloride casings, and preheated in a water bath at 40 °C for 1 h and followed by 90 °C for 30 min. After that, the gel was removed from water bath and immediately cooled down in ice for 10 min and stored at 4 °C before the measurements.

### Determination of gel strength

Gel strength of surimi gel was measured using a TA-XT plus texture analyzer (the Stable Micro System, London, UK) according to the described method (Guo, Li, Wang, & Zheng, 2019). The prepared gel (2 cm-high cylindrical specimens) was punctured using a cylindrical probe (P/0.5 in.) at a 50 % initial height distance. The pretest speed was set to 0.2 mm/s, and the speed of test and post-test were set to 1.0 mm/s with a 0.05 N trigger force. The resulting breaking force (g) multiplied deformation distance (mm) to obtain gel strength (g⋅mm). To facilitate the comparison of the changes of gel strength, the relative values (%) defined as the ratio of gel strength after and before the frozen storage were used in this study.

### Microstructural analysis

According to a previously reported method (Nian, Cao, & Cai, 2020), the surimi gels were cut into small blocks (3 × 3 × 3 mm) and immersed in 2.5 % glutaraldehyde solution at 4 °C for 24 h. The samples were then washed with phosphate buffer (0.2 M, pH 7.2) and deionized water. The fixed samples were dehydrated in a graded series of ethanol (30 %, 50 %, 70 %, 80 %, 90 %, 100 %, 10 min each), and then freeze-dried. Finally, the dried sample was placed on a stub and sprayed with gold for 60 s. The specimens were observed with a scanning electron microscope (Nova Nano SEM 230, FEI Co. ltd., USA) at an acceleration voltage of 6 kV.

### Statistical analysis

All the experiments were performed in triplicate and the data were presented with means ± standard. Statistical analysis was performed using Statistical Product Service Solutions (IBM SPSS Statistics 23.0, IBM, USA). One-way analysis of variance (ANOVA) with Duncan’s multiple range test and Student’s t-test were used to compare the difference between groups, and p < 0.05 was considered as significant difference.

### Results and discussion

#### Effect of NCC on salt soluble protein content

Myofibrillar protein is the dominating salt-soluble protein in surimi. The formation of hydrogen or hydrophobic bonds, disulphide bonds and salt bonds of myofibrillar proteins tend to occur in the frozen storage process, causing the changes of regular spatial structure of internal intermolecular and protein aggregation (Gao, Hou, & Zeng, 2019). The protein solubility has therefore been commonly found sharply decreased after frozen storage (Wu et al., 2014). In this study, the contents of salt soluble protein of surimi gradually decreased with the extension of the storage time (Fig. 1A), while significant differences in the salt soluble protein content were noticed between the negative control and those groups treated with NCC and sucrose/sorbitol (p < 0.05). In the NC group, the relative salt soluble protein content was quickly decreased to 76.83 % after 12 days, and finally decreased to 38.14 % at the end of storage, while that of 2 %, 4 %, 6 % and 8 % NCC-treated surimi was 54.42 %, 61.97 %, 73.57 % and 75.85 %, respectively. Furthermore, 6 % and 8 % of NCC addition exhibited better preservation effects on the salt soluble protein content, which were higher than that of PC (63.88 %, p < 0.05). These results suggested that the addition of NCC effectively retarded the decrease of the salt-soluble protein content of surimi and thereby improved the protein stability and surimi quality during frozen storage.
Effect of NCC on Ca$^{2+}$-ATPase activity

During the frozen storage process of surimi, the conformation changes and aggregation of globular myosin head of myofibrillar protein occur due to the growth of ice crystals and increases of the ionic strength, leading to a significant decrease in Ca$^{2+}$-ATPase activity (Tao et al., 2020). The activity of Ca$^{2+}$-ATPase therefore has been widely used for indicating the quality and the structural integrity of myofibrillar protein. In the 60-day frozen storage, the relatively Ca$^{2+}$-ATPase activity in the NC group without adding any cryoprotective substance was found to be dramatically decreased to 43.66 % as seen in Fig. 1B, suggesting the myofibrillar proteins were apparently denatured without protection. Meanwhile, both sucrose/sorbitol (8 %) and NCC (2 %-8%) were found to inhibit the quick decrease of the Ca$^{2+}$-ATPase activity in the frozen surimi samples compared to that of the control. Moreover, with increasing concentrations of NCC applied, the protective effect on the structural integrity of myofibrillar protein was stronger, leading to higher level of Ca$^{2+}$-ATPase activity. The addition of 8 % NCC demonstrated significantly higher Ca$^{2+}$-ATPase activity (82.85 %) than that of positive control (63.88 %) after the 60 days of storage. Addition of 4 % NCC resulted in a similar effect as that observed with an 8 % concentration of a commercial cryoprotectant. Effect of NCC on Ca$^{2+}$-ATPase activity might be partly explained by the hydroxyl groups presented on its surface, which tend to interact with water molecules in the surimi (Mu et al., 2019), thereby slowing ice crystal growth and protecting myofibrillar protein (Chen et al., 2022).

Effect of NCC on sulfhydryl content

The sulfhydryl group is the most active group in fish protein, which can be easily oxidized during frozen storage, causing the decrease of the sulfhydryl content and protein aggregation (Wu et al., 2014). Meanwhile, sulfhydryl groups are necessary for ATPase activity, therefore the oxidation of these groups also inactivates the Ca$^{2+}$-ATPase activity (Nopianti et al., 2012). As seen in Fig. 2, the changes of sulfhydryl contents during the frozen storage were in agreement with those in the Ca$^{2+}$-ATPase activity. The sulfhydryl contents of all groups decreased as the storage time increased, while the NC group showed the most significant decrease of sulfhydryl content from 6.54 mol/10 g to 2.93 mol/10 g. With the increase of NCC addition, the stronger inhibitive effects on the decrease of sulfhydryl contents in the NCC-treated groups were noticed during the storage. In particular, at the end of the storage, 3.85, 4.24, 5.13 and 5.09 mol/10 g of sulfhydryl contents were respectively observed in the groups with 2 %, 4 %, 6 % and 8 % NCC addition. It can be speculated that NCC might protect the ordered structure of protein and thus retard the exposure of sulfhydryl groups, preventing further oxidation of sulfhydryl groups (Xu et al., 2021). Moreover, NCC could also inhibit ice crystal growth and recrystallization to enhance protein integrity, reducing the degree of protein oxidation (Nian et al., 2020).

Effect of NCC on degradation of myofibrillar proteins

The SDS-PAGE profile of surimi samples before and after frozen storage was shown in Fig. 3. Myosin heavy chain (MHC) and actin are the major proteins bands in the studied samples, which are associated with the physiological function of myofibrillar protein. After the frozen storage, the significantly decreased band intensity of MHC and actin was observed in the negative control, indicating the severe degradation of these proteins (Zhang et al., 2018). The surimi proteins treated with sucrose/sorbitol (8 %) or NCC (2 %-8%) were effective in preventing the degradation of myosin and actin during frozen storage, and thus protecting the structural integrity and function of myofibrillar protein. With increase of NCC concentration, the stronger band intensity of MHC and actin was noticed. Based on the current findings, NCC could exhibit a marked protective effect on the stability of surimi proteins during the frozen storage, which also supports the results regarding the protein solubility, the Ca$^{2+}$-ATPase activity and sulfhydryl content.

Effect of NCC on secondary structure of myofibrillar proteins

FT-IR has been used as a powerful method to analyze the relative changes of protein secondary structure, which is strongly related to its biochemical properties and function. The protein secondary structures are mainly comprised of α-helix, β-turn, β-sheet and random coil structures (Sun, Luo, Cao, & Pan, 2016). The changes of secondary structure of myofibrillar proteins by incorporating NCC are represented in Fig. 4. During 60-day frozen storage, the proportions of α-helix in all samples were decreased, while other protein structures increased. In the NC group, α-helix proportion decreased drastically from 73.6 % to 31.2 % after frozen storage due to lacking of protection. Previous investigations also found that prolonged frozen storage significantly reduced α-helix proportion and affected the stability of protein secondary structures (Walayat et al., 2021; Walayat et al., 2020). However, in the surimi samples incorporated with 2 %-6% of NCC, the α-helix proportions at day 60 were 40.09 %, 43.45 % and 49.21 %, respectively, which were all significantly higher than that of surimi without any cryoprotectant (30.74 %). When the NCC addition was higher than 6 %, the protein structural constituents were basically similar. Meanwhile, the α-helix proportion of positive control at day 60 was similar to that of 4 % NCC group and lower than that of 6 % and 8 % NCC groups. The results suggested that 6 % NCC addition could significantly inhibit the decrease of α-helix proportion and improve the protein structural stability due to
its protective effects on the myofibrillar protein during frozen storage.

**Effect of NCC on surimi gel strength**

Surimi gel is a three-dimensional myofibrillar protein network, and gel strength is an important functional property determining the commercial quality of surimi products (Wei et al., 2018). However, myofibrillar proteins are vulnerable to freeze-induced denaturation, causing the decrease of gel strength (Tao et al., 2020). As shown in Fig. 5, the surimi gels incorporated with NCC or sucrose/sorbitol exhibited higher gel strength than that of surimi without any cryoprotectant. After 60 days of frozen storage, the relative gel strength of surimi in the control group showed the loose network structure with increasing number of large pores (Fig. 6d). Adding of NCC although did not significantly reduce the number of holes in gel structure, it could make a contribution to the formation of smaller holes. Therefore, surimi gel with NCC exhibited a compact microstructure with dense surfaces compared to the control (Fig. 6e). Based on our results, during frozen storage, NCC was able to prevent drastic protein denaturation and aggregation and inhibit the physical damage to the protein structure through inhibiting the growth of ice crystals (Li, Zhao, Zhong, & Wu, 2019). The integrity of protein structure plays an important role on the gel-forming capacity and gel strength. Moreover, due to its nano size, NCC could fill the protein network structure and exhibit a good compatibility with the protein matrix (Guo et al., 2018). These results revealed that NCC has great potential as a cryoprotectant to reduce the protein structure damage of surimi during frozen storage and also improve the surimi gel quality.

**Conclusion**

During a 60-day frozen storage, NCC exhibited a promising protective effect on the overall quality of surimi. Addition of NCC to surimi greatly inhibited the denaturation of myofibrillar protein, preventing the quick decrease of salt soluble protein content, Ca\(^{2+}\)-ATPase activity and sulfhydryl content of myofibrillar protein. Moreover, due to the hydroxyl groups presented on its surface, NCC tends to bind with water molecules in the surimi, thereby slowing ice crystal growth and recrystallization to enhance protein integrity, reducing the degree of protein oxidation. After frozen storage, non-denatured proteins show improved structure and gel strength. Our results indicated that NCC could not only serve as a nano-sized dietary fiber in surimi-based products, but also potentially serve as a novel cryoprotectant without the drawbacks to taste and caloric value of conventional cryoprotectants.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Fig. 6. Effect of NCC on microstructures of surimi gel during frozen storage.

Data availability

Data will be made available on request.

Acknowledgments

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