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Effect of chitooligosaccharides on the denaturation of weever myofibrillar protein during frozen storage

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ABSTRACT

We investigated the effect of chitooligosaccharides on the denaturation of weever (*Lateolabrax japonicus*) myofibrillar protein during frozen storage at −18 °C for 90 days. Chitooligosaccharides (2.5–10 g dry weight) were added to 100 g of myofibrillar protein, and the changes in the Ca-adenylpyrophosphatase (ATPase) activity, unfrozen water content, solubility, and sulfhydryl content of the myofibrillar protein were examined during frozen storage. We observed that the Ca-ATPase activity and solubility of the myofibrillar protein decreased gradually during frozen storage at −18 °C following the addition of chitooligosaccharides. In contrast, the Ca-ATPase activity and solubility of the myofibrillar protein of the control group decreased markedly during the first 45 days of storage and then further decreased gradually for up to 90 days of storage, indicating a biphasic denaturation pattern. The addition of chitooligosaccharides resulted in a significant increase in unfrozen water and sulfhydryl contents of the myofibrillar protein of the treatment group compared with that of the control group ($p < 0.05$) during frozen storage at −18 °C.

1. Introduction

Deterioration of marine products after catch occurs rather quickly compared with other food materials because of their high moisture and protein content, soft organization, and high bacteria-carrying capacity. Freezing is widely used for long-term preservation of fish, although the denaturation of fish protein occurs even during frozen storage and the quality of fish products deteriorates. Various sugars, amino acids, organic acids, phosphates, poly-alcohol proteolytic squid protein hydrolysate, and chitooligosaccharides are added to fish meal to avoid such unwanted changes and to reduce fish protein denaturation [1–5].

Chitooligosaccharides, oligomers of β-1,4-linked 2-amino-2-deoxy-D-glucopyranose (GlcN) and 2-acetamido-2-deoxy-D-glucopyranose, have numerous special biological, chemical, and physical properties, such as antifungal, antibacterial, and antitumour activities, which differ from those of ordinary chitosan [6]. Several studies have been conducted to demonstrate the effectiveness of chitooligosaccharides in preventing fish protein denaturation during frozen storage. Wang et al. observed that chitooligosaccharides suppressed the freeze-denaturation of actomyosin in *Aristichthys nobilis* surimi during frozen storage [5], whereas Xie and Timasheff reported the thermodynamic mechanism of protein stabilization by chitooligosaccharides [7]. However, it remains unknown whether chitooligosaccharides can reduce the denaturation of weever (*Lateolabrax japonicus*) myofibrillar protein during frozen storage.

In the present study, we investigated the protective effect of chitooligosaccharides against the freeze denaturation of weever myofibrillar protein. The effects of chitooligosaccharides on the Ca-adenylpyrophosphatase (ATPase) activity, solubility, unfrozen water, and sulfhydryl content of weever myofibrillar protein were examined.

2. Methods and materials

2.1. Materials

Live cultured weevers with a mean weight of 511.34 ± 51.07 g were purchased from an aquatic product market in Xinpu, China. Chitosan was purchased from Nanning Zhongnuo Biological Engineering Co., Ltd., China. All other chemicals were of reagent grade.

2.2. Chitooligosaccharides preparation

The chitooligosaccharides were prepared as described by Wu, but with a slight modification [8]. Briefly, chitosan was dissolved in 1% (v/v) aqueous acetic acid to a concentration of 1% (w/w) and the

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pH was adjusted to 5.0 using 1 M NaOH. A 20 mg mass of α-amylase was added into a reactor containing 500 mL of chitosan solution and then maintained in a thermostatic water bath at 50 °C for 4 h. The hydrolysates were neutralized with 1 M NaOH, filtered, concentrated to 16% (w/v), and precipitated with 5 volumes of ethanol. The precipitate was then filtered through a preweighed Whatman GF/A filter, dried at 60 °C for 3 h, and finally crushed.

2.3. Preparation of fish myofibrillar protein

The fish myofibrillar protein (Mf) was prepared according to the method as described by Hossain et al., but with a slight modification [1]. The fish were killed by a blow to the head, scaled, gutted, and then the heads were chopped off. Fresh weever muscles were cut into thin sections and washed 3 times with 5 volumes of 0.1 M KCl–20 mM Tris-maleate buffer (pH 7.0). Three volumes of KCl–Tris-maleate buffer were added to the muscles, and the specimens were homogenized using a homogenizer (GF-1, Beijing Zhongyi Zhonghe Biotechnology Co., Ltd, Beijing, China). The homogenate was filtered through nylon net (#16) to remove the connective tissues, after which 20% Triton X-100 was added to the homogenate to adjust its final concentration to 1%. The homogenate was allowed to stand for 30 min and centrifuged at 750 × *g* for 10 min. After centrifugation, the sediment was washed with five volumes of KCl–Tris-maleate buffer and centrifuged once more. This procedure was repeated four times.

Then the sediment was mixed with 5 volumes of cold distilled water, washed by stirring, and centrifuged at 4000 × *g* for 10 min. The sediments were further centrifuged at 25,000 × *g* for 20 min. All the procedures were performed at 5 °C.

0.25–1 g dry weight was added to 100 g of the Mf sample. The pH of the mixture was then adjusted to 7.0 with 0.01 N NaOH or 0.01 N HCl. After mixing at 5 °C for 15 min, about 3 g of the sample was sealed in a test tube (inner diameter 12 mm and length 75 mm) and stored at −18 °C. As a control treatment, Mf without chitooligosaccharides was stored in the same manner.

2.4. Ca-ATPase activity determination

The myofibril samples were thawed in a cold room at about 5 °C after various periods of storage at −18 °C. The samples were homogenized in 30 parts of 0.1 M KCl – 20 mM Tris-maleate buffer (pH 7.0), and the homogenate was centrifuged at 750 × *g* for 10 min. The sediment was washed with KCl–Tris-maleate buffer and centrifuged further at 750 × *g* for 10 min. This procedure was repeated twice, and the obtained sediment was re-suspended in the buffer [9].

Myofibrillar Ca-ATPase activity was determined by the following method [10]. Myofibril protein samples of 0.2–0.4 mg were incubated at 25 °C in the presence of 100 mM KCl, 5 mM $CaCl_2$, 25 mM Tris-maleate (pH 7.0) and 1 mM adenosine triphosphate, and the reaction was terminated by addition of 30% trichloroacetic acid to a final concentration of 5%. The inorganic phosphate liberated in the supernatant was measured using the method of Fiske and Subbarow [11]. Specific activity was expressed as micromoles of inorganic phosphate released per milligram of protein per minute, and the Ca-ATPase activity of the frozen myofibrils was expressed as the ratio of the specific activity before freezing (relative%).

2.5. Measurement of unfrozen water

The amount of apparent unfrozen water considered as the bound water and partially bound water in the frozen Mf was measured according to the previously reported method using differential scanning calorimeter (DSC) (model SSC-5200, Seiko Electronic Industry Inc., Tokyo, Japan), to assess the changes of state of water in Mf after addition of chitooligosaccharides, and the stability of Mf during long term freezing [1]. The heat of fusion of distilled water (5–25 mg) was measured initially to establish a linear relationship between the amount of pure water and the heat of fusion. The heat of fusion of distilled water (5–25 mg) was 79.2 cal/g. Twenty milligrams Mf was placed in a tightly sealed aluminum cell and accurately weighed, and 20 mg of Al_2O_3 was sealed in another aluminum cell used as reference. The cells were subjected to DSC analysis where the heat of fusion was measured by rising the temperature from −40 to 25 °C at a rate of 1 °C per min. The measured endothermic peak area reflected the heat of fusion necessary to melt the ice, which corresponded to the free water content in Mf. The peak melting temperature was observed at −2.30 to −2.37 °C for the control and the peak points were shifted gradually with the increase of added-concentration of in the Mf (−2.73 to −5.60 °C). After perforation, the cells containing Mf were dried at 105 °C for 24 h to determine the total water content in the Mf. The apparent unfrozen water was determined by deducting the free water from the total water content in the sample.

2.6. Salt solubilization of Mf determination

Mf solutions at different stages of freezing were centrifuged (9000 × *g*, 4 °C) for 50 min. The biuret protein concentration in the supernatant was determined, and the protein concentration in the solution representing the percentage of the initial protein concentration of the myofibrils was referred to as soluble salts.

2.7. Sulfhydryl contents in myofibrillar protein determination

Sulfhydryl groups were determined according to the method described by Soyer, Özalp, Dalmış and Bilgin [12]. The total concentration of free sulfhydryl groups was determined by observing the reaction of the proteins with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). One gram of meat was blended with 50 mL of cold distilled water and homogenized. The protein in the homogenate was diluted to 2 mg/mL with 0.1 M phosphate buffer (pH 7.4), and protein content was determined using the biuret method. About 0.5 mL of the homogenate was transferred to a tube and dissolved in urea buffer (1:1). After incubation with 0.5 mL of DTNB reagent at room temperature for 15 min, the absorbance of the solution was measured at 412 nm. Sample blanks with 0.5 mL of phosphate buffer without DTNB and reagent blanks with only water were prepared. Sulfhydryl content was calculated using a molar extinction coefficient of 11,400 M^{-1} cm^{-1} for 5,5′-dithiobis at this wavelength. Results were expressed as nmol of total free sulfhydryl groups per milligram of protein.

2.8. Analytical methods

The pH of the solution was recorded using a digital pH meter (Model: PHS-3C, CD Instruments, China). Ash, moisture, and total sugar content of the samples were determined per standard methods) [13]. Protein concentrations were determined using the biuret method [14], in which bovine serum albumin was used as a standard. The composition of the sugar in hydrolyzed chitosan was analyzed by Water600 HPLC equipped with a double column system. The first column (Sugarpark 1, 6.5 mm i.d. × 300 mm) used pure water as mobile phase at a flow rate of 0.5 ml/min and the column temperature was maintained at 85 °C. The second column (Spherisorb NH_2, 4.6 mm i.d. × 250 mm) used acetonitrile/water (70/30, v/v) as mobile phase at a flow rate of 1 ml/min and the column temperature was 30 °C. The detector sensitivity was 4 and the inject volume was 10 μl.