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Effect of trehalose on *Lateolabrax japonicus* myofibrillar protein during frozen storage

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ABSTRACT

The effect of trehalose on the denaturation of weever (*Lateolabrax japonicus*) myofibrillar protein during frozen storage at −18 °C for 90 d was investigated. Trehalose (2.5–10 g dry weight) was added to 100 g of myofibrillar protein, and changes in the Ca^{2+}-adenylpyrophosphatase (ATP_{ase}) activity, solubility, sulfhydryl content, and unfrozen water content of myofibrils were examined during frozen storage. Ca^{2+}-ATP_{ase} activity and myofibrillar protein solubility decreased gradually during frozen storage at −18 °C upon addition of trehalose. By contrast, Ca^{2+}-ATP_{ase} activity and myofibrillar protein solubility in the control group dropped drastically during the first 45 d of storage and then further decreased gradually for up to 90 d of storage, indicating a biphasic denaturation pattern. Trehalose addition significantly increased sulfhydryl and unfrozen water contents in the myofibrillar protein of the treatment groups compared with that of the control group ($p < 0.05$) during frozen storage at −18 °C.

1. Introduction

Freezing is a widely used method for the long-term preservation of fish. However, fish proteins are prone to freeze denaturation during frozen storage, which results in deterioration of fish quality. Freeze denaturation reduces fish protein functions, such as fish meal hardening, water holding capacity, protein solubility, and gel-forming ability, and others. To address these issues, sugars, amino acids, organic acids, phosphate and poly-alcohol proteolytic squid protein hydrolysate (Hossain et al., 2004; Matsumoto & Arai, 1987; Nozaki, Ichikawa, & Tabata, 1991; Ooizumui, Hashimoto, Ogura, & Arai, 1981) are commonly added to fish meal to reduce protein denaturation.

Trehalose, α-D-glucopyranosyl-(1 → 1)-a-D-glucopyranoside, is a carbohydrate found in living organisms. It is a non-reducing glucose disaccharide commonly detected at high concentrations in anhydrobiotic organisms (Xie & Timasheff, 1997). Trehalose has a potential biotechnological importance because of its effectiveness in stabilising membrane structures in the dry state, inhibiting biological damage at low temperatures and stabilising protein structures during freezing and freeze-drying (Hedoux, Paccou, Achir, & Guinet, 2013; Jain & Roy, 2010; Zhang, Liu, Dong, & Sun, 2012).

The weever (*Lateolabrax japonicus*) aquaculture industry has developed rapidly over the last few years, and fish production has recently increased. The common practice of selling live fish, however, has exerted great pressure on fish farmers to meet growing demands. As such, the preservation and processing of bass fish are major concerns for the fish processing industry.

In this study, the protective effect of trehalose against the freeze denaturation of weever (*L. japonicus*) myofibrillar protein was investigated. The optimum trehalose concentration was determined, and the effects of trehalose on the Ca^{2+}-adenylpyrophosphatase (ATP_{ase}) activity, solubility, sulfhydryl content, and unfrozen water content of weever (*L. japonicus*) myofibrillar protein were investigated.

2. Methods and materials

2.1. Materials

Live cultured weevers with a mean weight of 507.34 ± 42.52 g were purchased from an aquatic product market in Xinpu, China. Trehalose was purchased from Nanning Zhongnuo Biological Engineering Co., Ltd., China. All other chemicals used were of reagent grade.

2.2. Preparation and frozen storage of myofibrils

Myofibrils were prepared according to the method of Yamashita, Zhang, and Nozaki (2003) with slight modifications. The fish were killed by a blow to the head, scaled, gutted, and then the heads were

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chopped off. Fresh weever muscles were cut into thin sections and washed three times, with five volumes of 0.1 M KCl–20 mM Tris-maleate buffer (pH 7.0) by stirring. Three volumes of KCl–Tris-maleate buffer were added to the muscles, and specimens were homogenised using a homogeniser (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.

To remove as much KCl-derived buffer from the sediment as possible, the sediment was mixed with five volumes of cold distilled water, washed by stirring and centrifuged at 5000*g* for 10 min. To remove excess water from the sediment, centrifugation was repeated (12,000*g*, 20 min), and the resulting sediment was used as the myofibril specimen. All procedures were performed at approximately 5 °C.

Trehalose (2.5–10 g dry weight) was added to 100 g of the myofibril sample. The pH of the mixture was then adjusted to 7.0 with 0.01 M NaOH or 0.01 M 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, myofibrillar protein without trehalose was stored in the same manner.

2.3. Determination of Ca^{2+}-ATP_{ase} activity

The myofibril samples were thawed in a cold room at about 5 °C after various periods of storage at −18 °C. The samples were homogenised 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 (Yamashita et al., 2003).

Myofibrillar Ca^{2+}-ATP_{ase} activity was determined by the following method (Katoh, Uchiyama, Tsukamoto, & Arai, 1977). Myofibril 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. 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 (1925). Specific activity was expressed as micromoles of inorganic phosphate released per milligramme of protein per minute, and the Ca^{2+}-ATP_{ase} activity of the frozen myofibrils was expressed as the ratio of the specific activity before freezing (relative%).

2.4. Determination of salt solubilisation of myofibrillar protein

Myofibrillar protein 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.

2.5. Determination of sulfhydryl content in myofibrillar protein

Sulfhydryl groups were determined according to the method described by Soyer, Özalp, Dalmış§, and Bilgin (2010). 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 homogenised. 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 ηmol of total free sulfhydryl groups per milligramme of protein.

2.6. 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 trehalose, and the stability of Mf during long term freezing (Hossain et al., 2004). 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 milligrammes Mf was placed in a tightly sealed aluminium cell and accurately weighed, and 20 mg of Al_2O_3 was sealed in another aluminium 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 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.

2.7. Protein concentration determination

Protein concentrations were determined using the biuret method (Robinson & Hodgen, 1940). Bovine serum albumin was used as a standard.

2.8. Statistical analysis

All data are presented as mean ± S.D. Statistical analysis was performed using Statgraphics Centurion XV version 15.1.02. Multifactor ANOVA with posterior multiple range test was used to determine significant differences between groups.

3. Results and discussion

3.1. Effect of trehalose concentration on the Ca^{2+}-ATP_{ase} activity of weever myofibrillar protein during frozen storage

The 3D structure of proteins, which determines their physiological activities, is formed and stabilised by hydrogen bonds, hydrophobic interactions and hydration of polar residues. However, proteins may be denatured when these bonds are disturbed, resulting in the loss of physiological activities. Freeze denaturation of fish protein is induced by high salt concentrations, changes