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Investigation of the physiochemical properties, cryoprotective activity and possible action mechanisms of sericin peptides derived from membrane separation

Ling Li [a], Jin-Hong Wu [b, *], Li Zhang [b], Xu Chen [a], Yan Wu [b], Jian-hua Liu [b], Xue-qing Geng [b], Zheng-Wu Wang [b], Shao-Yun Wang [a, **]

[a] Institute of Food and Marine Bioresources, College of Biological Science and Technology, Fuzhou University, Fuzhou 350108, China
[b] Department of Food Science and Engineering, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China

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ABSTRACT

In order to exploit their industrial applications, sericin peptides (3K-SP) were obtained by membrane separation and their physiochemical properties and cryoprotective function were investigated. Results showed that 3K-SP were mostly distributed less than 3000 Da, and rich in the amino acids Ser, Asp, Gly, Thr and Glu, which have been associated with the cryoprotective activity of ice-structuring proteins. Addition of 3K-SP to a frozen solution led to the reductions in melting temperature and melting time compared to control solution. In addition, 3K-SP inhibited ice recrystallization, since it could maintain small ice crystal sizes within a frozen solution. Furthermore, 3K-SP demonstrated high cryogenic protection activity of *Lactobacillus delbrueckii* Subsp. *Bulgaricus* during freezing, and provided optimal protection of cells at conditions in which its concentration was 1.0 mg/mL and the pH of the solution was 7.0. In these conditions, the percentage of surviving cells was as high as 84.78 ± 3.07%. Flow cytometric and scanning electron microscopy analyses showed that treatment with 3K-SP increased the percentage of viable cells from 52.9% to 80.1%, and suggest that 3K-SP may mediate its protective effects through interaction with cell membranes, whereby it surrounds cells in a glassy matrix that helps maintain their membrane integrity.

1. Introduction

Antifreeze proteins (AFPs) have been identified in a number of different bacteria, plants, fish and insects that live at sub-zero temperatures, where they function in these organisms to prevent uncontrolled ice growth, which can be lethal (Devries, Komatsu, & Feeney, 1970; Devries, 1983; Duman & DeVries, 1972; Komatsu, DeVries, & Feeney, 1970; Feeney, 1974; Sally & Yu, 2010). AFPs have attracted a great deal of interest in the scientific and industrial communities for their ability to prevent cryoinjury upon exposure to cold temperatures (Venketesh & Dayananda, 2008). AFPs are a complex class of biological antifreezes that possess significant structural heterogeneity, which has made it difficult to understand the structural underpinnings of their cryoprotective activity. Despite these challenges, AFPs have been found to exhibit two major types of antifreeze activities. One of these activities, thermal hysteresis (TH), is defined as the selective depression of the freezing point of a solution relative to its melting point and is the result of the binding of AFPs to the surfaces of ice crystals in the solution (Wilson, 1993). The other antifreeze activity is known as ice recrystallization inhibition (IRI), and functions by keeping ice crystals in small shapes within a frozen sample (Tomczak, Marshall, Gilbert, & Davies, 2003). These two distinct types of antifreeze activities have been exploited for the cryoprotection of diverse substances. AFPs prevent ice recrystallization in frozen foods such as ice cream and frozen meats (Damodaran, 2007; Li & Sun, 2002) and act as protecting agents in the cryogenic or hypothermic storage of biological materials, such as cells or tissues, by inhibiting formation of large ice crystals and thereby preventing leakage of ions from cell membranes (Venketesh & Dayananda, 2008). Although AFPs are used in many products, the difficulties

* Corresponding author.
** Corresponding author.
E-mail addresses: wujinhong@sjtu.edu.cn (J.-H. Wu), shywang@fzu.edu.cn (S.-Y. Wang).

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associated with producing large quantities of AFPs have limited their use in industrial applications.

Sericin is a water-soluble protein that is created by silkworms in the production of silk. In silkworm cocoons, sericin coats fibers of fibroin, another major component of silk, allowing the fibroin proteins to adhere to one another. Sericin is used as a biomaterial because of its antibacterial and UV resistant properties (Zhang, 2002). In recent studies, sericin and its peptides have been shown to possess the ability to bind ice (Wu, Zhou, Wang, Wang, Wu, & Guo, 2013; Wu, Rong, Wang, Zhou, Wang & Zhao, 2015), properties that have led to the use of sericin as a supplement in serum-free freezing medium, which is used to cryopreserve mammalian cells, human hepatocytes, and bovine embryos (Miyamoto, Teramoto, Hayashi, & Enosawa, 2010; Sasaki, Kato, Yamada, & Terada, 2005; Tomohiro & Yoshihisa, 2013). These previous findings suggest that sericin and its peptides are novel and effective cryoprotectants. At present, although products containing AFP or antifreeze glycoproteins (AFGP) are commercially available, their high prices have limited their uses to research and other special applications.

Sericin ice-binding peptides have been isolated previously at a small, laboratory scale (Wu et al.,2013). Ice-binding sericin peptides purified by ice affinity extraction adsorption are small, with most possessing molecular weights of less than 2000 Da. To purify sericin peptides with cryoprotective activity more easily and at a larger scale, membrane separation, a process that is normally applied in the industrial preparation of biomaterials, was performed in this study. The products of the membrane separation represented a novel biomaterial, and were further investigated for their physiochemical properties.TH and IRI activities were measured by nuclear magnetic resonance (NMR) micro-imaging and microscopy at low temperature. The products were also assessed for their ability to protect *Lactobacillus* cells from hypothermia. Finally, a possible structural mechanism by which the products prevent freezing was determined by scanning electron microscopy (SEM) and multiparametric flow cytometry.

2. Materials and methods

2.1. Materials

The *Lactobacillus* used throughout the study was a *Lactobacillus delbrueckii* Subsp. *Bulgaricus* LB340, also known simply as "*L. Bulgaricus*", and was purchased from Danisco (CN) Holding Co. Ltd. (Shanghai, China). M17 broth was purchased from Qingdao Hope Bio-Technology Co. Ltd (Tsingtao, China). Hydrolyzed sericin powders with more than 98% peptide content and prepared by Neutrase (Novozymes Investment Co. Ltd, China) hydrolysis were obtained from Huzhou Xintiansi Bio-Tech Co. Ltd. (Huzhou, China), cFDA was purchased from (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China), and PI was purchased from (Sigma–Aldrich Co. Ltd, Lyon, France). All other reagents were of analytical grade.

2.2. Preparation of sericin peptides

Hydrolyzed sericin powders were dissolved in distilled water to make a 5% solution. The solution was used for subsequent offline membrane separation performed by an ultrafiltration unit consisting of an ultrafiltration device (Shanghai Mosu Science Equipment Co. Ltd, Shanghai, China) equipped with a 3 kDa polysulfone membrane. Peptides in the solution with molecular weights of less than 3 kDa were separated from other macromolecular components using continuous membrane separation at a constant pressure of 0.4 Mpa at room temperature (25 °C). The permeate fluids were collected and freeze-dried as sericin peptides (designated as 3K-SP) for subsequent analysis. The yield of 3K-SP was calculated as follows:

$$\text{The yield of } 3K-SP\ (\%) = \frac{A_1}{A_0} \times 100$$

where A_1 was the weight of dried 3 k-sericin peptides (g) and A_0 was the weight of hydrolyzed sericin powders (g).

2.3. Analysis of amino acid composition and molecular mass distribution of 3K-SP

The amino acid composition of 3K-SP was analyzed by the L-8900 amino acid automatic analyzer (Hitachi, Japan) according to the method of Wang et al. (2012) with minor modifications. Samples were hydrolyzed using 6 mol/L HCl at 110 °C for 24 h under vacuum. The hydrolysate was dried, dissolved in 0.02 mol/L HCl, and centrifuged at 10,000 rpm for 20 min, and the collected supernatants were filtered through 0.22 μm membrane prior to injecting into the amino acid analyzer. Amino acid compositions were then obtained using an automatic analysis algorithm in the amino acid automatic analyzer by calibrating with standard amino acids.

The molecular mass distribution of 3K-SP was determined by high performance liquid chromatography with a Waters™650E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA), the detailed procedures were as described previously by Wu et al. (2013).

2.4. Nuclear magnetic resonance (NMR) micro-imaging experiment

To determine the effects of 3K-SP on the melting of frozen solution, 2.0 mg/mL and 5.0 mg/mL of 3K-SP were prepared in distilled water. Distilled water was used as a negative control and arginine (2.0 mg/mL, Arg) was used as a positive control. Glass tubes (inner diameter = 15 mm) holding 5.0 mL of each solution were frozen in a −20 °C freezer overnight for NMR micro-imaging experiments.

NMR micro-imaging experiments were carried out with a MiniQMR NMR micro-imaging system (Shanghai Niumag Corporation, China). The pre-frozen sample tubes holding the frozen solutions were quickly transferred inside the resonator. A multislice multispin-echo sequence was used to image the mobile water in the samples. Three slices with a slice thickness of 3.0 mm and an interslice distance of 3.0 mm were measured, and the intermediate slices, which had a field of view of 3.5 cm in the axial direction, were used. Other parameters included echo time = 18.2 ms,

Table 1
Amino acid composition of 3K-SP.

Amino acid	Content (%, w/w)
Ser	26.14
Asp	15.83
Gly	8.88
Thr	7.51
Glu	6.89
Ala	3.63
Lys	3.24
Val	2.55
Arg	1.81
Leu	1.22
Tyr	1.15
His	1.13
Ile	1.06
Phe	0.34
Cys	0.14
Met	0.13

Values are means from duplicate analysis.