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Salting-in effect on muscle protein extracted from giant squid (*Dosidicus gigas*)

Rui Zhang [a], Ru Zhou [a], Weichun Pan [a,*], Weiwei Lin [a], Xiuzhen Zhang [a], Mengya Li [a], Jianrong Li [b], Fuge Niu [a], Ang Li [a]

[a] Food Safety Key Laboratory of Zhejiang Province, The School of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310018, China
[b] College of Chemistry, Chemical Engineering and Food Safety, Province Key Laboratory of Food Quality Safety and Functional Food, Bohai University, Jinzhou, Liaoning 121013, China

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ABSTRACT

The salting-in effect on muscle protein is well-known in food science but hard to explain using conventional theories. Myofibrillar protein extracted from the giant squid (*Dosidicus gigas*) was selected as a model muscle protein to study this mechanism in KCl solutions. Changes in the secondary structures of myofibrillar protein molecules caused by concentrated salts, particularly in the paramyosin molecule conformation, have been reported. Zeta-potential determinations showed that these secondary structures have modified protein molecule surfaces. The zeta-potential of the myofibrillar protein molecules fell from -7.24 ± 0.82 to -9.99 ± 1.65 mV with increasing salt concentration from 0.1 to 0.5 M. The corresponding second virial coefficient increased from $-85.43 \pm 3.8 \times 10^{-7}$ to $-3.45 \pm 1.3 \times 10^{-7}$ mol mL g^{-2}. The extended law of corresponding states suggests that reduced attractive interactions increase the protein solubility. Solubility measurements in alternating KCl concentrations showed that the conformational change was reversible.

1. Introduction

KCl is an additive commonly used to reduce the amount of NaCl used in food because of health concerns (Grummer, Bobowski, Karalus, Vickers, & Schoenfuss, 2013). High daily salt intakes are a major cause of cardiovascular disease (Zandstra, Lion, & Newson, 2016). This can be addressed by replacing NaCl with KCl to maintain product taste. However, although much is known about the effects of NaCl concentration on food protein, which have been studied for a long time, few results are available on the effects of KCl. This is because thermodynamically KCl has been treated as NaCl with minor differences. However, the abilities of KCl and NaCl to destroy protein molecule stability differ because their cation sizes differ (Jungwirth & Cremer, 2014). The effects of KCl concentration on food protein stability therefore need to be studied.

These compounds have two functions, i.e., adjusting flavors and regulating interactions between macromolecules (Israelachvili, 2011). The simplest and commonest method for assessing intermolecular interactions is solubility determination (Atkins, 1998). Food protein solubility also reflects the functional properties of proteins, one of main nutrients in food (Tapal & Tiku, 2012). Protein solubility data are therefore widely used in the food industry to assess food quality and optimize food processing. In protein solutions, the salting-in effect usually occurs at low salt concentrations, at which there is an affinity between the ions and the protein molecule surfaces. As a result, the protein molecule surfaces becomes more charged with increasing salt concentration, and an increasingly repulsive double-layer force dominates the intermolecular interactions (Bockris, Bowler-Reed, & Kitchener, 1951). According to the extended law of corresponding states (Noro & Frenkel, 2000), protein solubility is enhanced, leading to the salting-in effect. However, as the salt concentration rises, the ions saturate the protein molecule surfaces and the charges on the protein molecules approach a constant level. At this point, the screening effect dominates and reduces the repulsive double-layer force, leading to a salting-out effect. However, this does not always happen. One well-known exception is muscle protein, which has a much wider salt concentration range than that predicted by conventional theories of the salting-in effect. Two types of muscle protein, extracted from giant squid (*Dosidicus gigas*) and pork (*Susdomesticus*), have been tested (Wu et al., 2016; Zhou et al., 2015). The results showed that the concentration range for the salting-in effect is up to 1 M, about 10 times higher than the upper bound reported by Bockris et al. (1951). These results are

* Corresponding author at: The School of Food Science and Biotechnology, Zhejiang Gongshang University, 18 Xuezhen Street, Hangzhou 310018, China.
E-mail address: panweichun1@yahoo.com (W. Pan).

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hard to explain using available theories such as those of Hofmeister (1888a, 1888b), and Aoki, Shiraki, and Hattori (2016). From a theoretical viewpoint, it is therefore worth investigating the mechanism of the salting-in effect. Protein, especially muscle protein, is widely used in the food industry to create a desirable food texture (Campbell, Euston, & Ahmed, 2016). Various salt concentrations are used to modify the protein solubility to regulate the formation of networks of protein molecules (Lantto, Puolanne, Kruus, Buchert, & Autio, 2007; Molina, Petruccelli, & Anon, 2004). Studies of the salting-in effect at the molecular level could therefore provide guidance for food industry practices such as process optimization and food product design.

Muscle proteins extracted from the giant squid are a suitable model for several reasons. First, conventional sources of fish have declined rapidly recently and the rate of expansion of the human population is unprecedentedly high. The nutritional supply, especially of protein, is critically insufficient in Africa and some other regions. The giant squid is rich in protein but has the shortcomings of poor flavor and high protein solubility (Sanchez-Brambilia, Alvarez-Manilla, Soto-Cordova, Lyon, & Pacheco-Aguilar, 2004), and these narrow its range of applications in the food industry. If these problems can be solved, giant squid could be one of the best available solutions for overcoming nutrient insufficiency. Although some progress has been made by the food industry, e.g., using salt, hydrocolloids, and temperature profiles to enhance the quality of giant squid protein, especially its gelation ability (Cortés-Ruiz, Pacheco-Aguilar, Elena Lugo-Sánchez, Gisela Carvallo-Ruiz, & García-Sánchez, 2008; Gómez-Guillén, Montero, Solas, & Borderías, 1998), the detailed mechanisms involved in these practices are still unclear. In addition, muscle is a good subject for scientific study. Muscle, which has a hierarchical and heterogeneous structure built from commonly available elements, has excellent physical properties that are hard to attain using current techniques. The study of muscle structure at the molecular level can guide the development of similar artificial materials and also provide insights into the self-assembly process.

Because of the multiple components present in muscle protein, the details of molecular structure evolution under various protein solution conditions differ. Information can be obtained from either protein molecular structure modification or by changing the solute composition. To verify the possibility of molecular structure modification when the solution conditions such as salt concentration change, muscle protein should be separated into its pure components. The same conditions should be used with these pure component solutions to check whether the protein structure has changed. Myosin is the most important muscle protein in our research, but we have not yet been able to purify this molecule. The effects of salt concentration on myosin are therefore not discussed in this study.

2. Materials and methods

2.1. Raw materials

Dead giant squid, which had been stored at −20 °C for less than 3 months after being caught and immediately being frozen without any further treatment, were obtained from the Zhoushan Second Marine Fisheries Company in Zhejiang, China. All squid (usually four organisms of average mantle size 1 m) were deheaded, degutted, and cleaned using water after they were defrosted in air to room temperature. The mantles with the fins were bagged and stored at −80 °C for further experiments.

Reagent grade Na_2HPO_4, KH_2PO_4, KCl, CH_3CH_2OH, and CH_3COOH were provided by Huipu Inc. (Hangzhou, China). All other reagent grade chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of myofibrillar protein

Myofibrillar protein samples were prepared using the procedure described by Hashimoto, Watabe, Kono, and Shiro (1979), with minor modifications. The muscle was minced using a meat grinder. A mixture of ground muscle and buffer A (15.6 mM Na_2HPO_4, 3.5 mM KH_2PO_4, pH 7.5) (1:10, w/v) was homogenized using a tissue homogenizer (Homogenizer T25 basic, IKA, Staufen, Germany) at 9600 rpm. Homogenization was performed in an ice bath to avoid overheating and consequent protein denaturation; five cycles of homogenization for 30 s followed by a 30-s interval were performed. The connective tissues were removed by filtration using two layers of gauze. The filtrate was incubated for 20 min at 4 °C and then centrifuged at 5000*g* for 15 min at 4 °C (Biofuge Stratos, Thermo Scientific Inc., Belmont, CA, USA). The supernatant was removed and the sediment was suspended in 10 volumes of phosphate buffer B (0.1 M KCl, 15.6 mM Na_2HPO_4, 3.5 mM KH_2PO_4, pH 7.5). This sample was centrifuged at 5000*g* for 15 min at 4 °C. This procedure was repeated three times. The final pellet (the myofibrillar protein precipitate) was blended with buffer A to 300 mL; aliquots were placed in 10-mL plastic centrifuge tubes and stored at −80 °C (Forma 702, Thermo Scientific Inc.).

2.3. Paramyosin separation and purification

An AKTA purifier 100 fast protein liquid chromatography system (GE Healthcare, Pittsburg, PA, USA) was used to purify the target proteins. The myofibrillar protein prepared as described in Section 2.2 was dissolved in phosphate-buffered saline (PBS) solution. The myofibrillar protein sample (6.25 mg mL^{-1}) was then loaded onto a HiTrap Q FF column (5 mL, GE Healthcare) that had been pre-equilibrated in PBS buffer. The column was washed with 0.1–1 M KCl using 42 column volumes (CVs) in three consecutive steps, with 0.3, 0.5, and 1 M KCl (Fig. 1). The protein concentration was measured based on ultraviolet (UV) absorption at 280 nm. The composition of each fraction (1 mL) was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions (T47, T59, T85, and T121) with similar protein compositions were pooled, desalted, and concentrated to 3 mg mL^{-1}.

Sample T47 (500 μL) was loaded onto a Superdex 200 10/30 GL gel filtration column (24 mL, GE Healthcare) that had been equilibrated with PBS buffer (0.1 M KCl, 15.6 mM Na_2HPO_4, 3.5 mM KH_2PO_4, pH 7.5) and then eluted with 1.5 CVs of this PBS buffer solution at a rate of 0.5 mL min^{-1} (Fig. 2). The 17th, 18th, and 19th fractions were desalted and concentrated. Part of the protein sample was used for assessment, and the rest was dried using a custom-built vacuum freeze-drying system; the obtained powder was packaged and stored at −20 °C.

2.4. Circular dichroism (CD) analyses of paramyosin

CD spectra were recorded in the far-UV range (250–190 nm) using a CD spectropolarimeter (J-815, Jasco Corp., Tokyo, Japan) and a 0.1-cm quartz CD cuvette at 25 °C. The protein concentration for analysis was maintained at 50 μg mL^{-1} for all samples; the corresponding solutions without protein were used as blank buffers. The scan rate, response, bandwidth, and sensitivity were set at 50 nm min^{-1}, 0.25 s, 1.0 nm, and 0.1°, respectively. Each spectrum was recorded in triplicate. The percentages of α-helix, β-sheet, β-turn, and random coil structures were assessed using the protein secondary structure estimation program provided with the Jasco J-815 spectropolarimeter (Whitmore & Wallace, 2004).