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Original article

Influence of the protease inhibitors on the gel-forming ability of recovered North Pacific krill *Euphausia pacifica* protein using NaCl solution treatment

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Introduction

North Pacific krill *Euphausia pacifica* widely inhabits the sea around north part of Japan. However, their utilization for human food consumption has been limited due to their small size and high endogenous enzyme activity [1,2]. Moreover, most of them are used for aquaculture feed and its commercial value is very low [3]. Fish gel products, such as *kamaboko*, has the highest production volume among the seafood in Japan [4]. Therefore, for the effective utilization of the krill as a raw material for gelled product, the krill protein was recovered in our previous study [5]. It was concluded that the NaCl solution treatment to recover the protein is useful method to obtain the protein with retaining its activities of Ca-ATPase and Mg-ATPase, which are indicators of protein functionality [6], and that the autolysis of the protein during recovering process could be suppressed by inhibiting serine protease and metalloprotease (Amano et al., unpubl. data). For the next step, the gel-forming ability of the recovered protein in the presence of protease inhibitors was evaluated in this study.

Thus, the objective of the present study was to clarify the effect of endogenous protease on the thermal gel-forming ability of the recovered krill protein. This study might provide a theoretical basis for future studies for utilization of krill protein as commercial gel-products.

Materials and methods

Sample preparation
North pacific krill were caught in the offshore from Sanriku district in Japan on 4 April, 2015 and transported to our laboratory on the next day. After the arrival, the krill was mixed with 7.5% (w/w) of sucrose as a cryoprotectant. Then, the krill was ground to mince by using a meat grinder (M-22A; Nantsune Co., Ltd., Osaka, Japan) with a 2.4 mm sieve. The minced krill was packed into sealed plastic bags and stored at -30°C until using.

Chemicals
Benzamidine hydrochloride hydrate (Benzamidine), and trypsin inhibitor from soybean (SBTI) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Phenylmethanesulfonylfluoride (PMSF) was purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA). Ethylenediamine-*N,N,N,',N'*-tetraacetic acid, trisodium salt, trihydrate (EDTA) was product of Dojindo Laboratories (Kumamoto, Japan).

Recovery of the protein from krill
The krill protein was recovered according to our previous study [5]. The minced krill was homogenized with pre-cooled 8.0% NaCl solution at a ratio of 1:1 (w/v) to solubilize the protein. The homogenate was shaken in ice for 10 min and centrifuged at 15,000×*g* for 10 min at 4°C to remove insoluble materials such as eyeball or shell. The supernatant was diluted to 10 times (v/v) by cold ion exchanged water, and then re-centrifuged to precipitate the protein. The precipitate was mixed with protease inhibitors (50 mM Benzamidine, 1.0 mg/g SBTI, 5 mM PMSF and 100 mM EDTA) whose inhibitory effect on autolysis of krill protein was confirmed in our previous study (Amano et al., unpubl. data). The mixture was dehydrated for 24 hour at 4°C with a dehydration sheet (Pichit sheet; Okamoto Industries, Inc., Tokyo, Japan) to obtain krill protein. Final moisture content of the protein was adjusted to 80% and mix with 7.5% sucrose, and then kept at -30°C until use for next step.

Preparation of thermal gel
Thermal gel from recovered krill protein was prepared according to the method of our previous study [5]. The recovered protein was ground using masticator (PS;

IUL, S. A., Barcelona, Spain) for 30 min with 3% NaCl and its final moisture content was adjusted to 80%. The ground protein was shaped in plastic tube (23.0 mm in diameter) and put into water bathes for 30 min at 40, 60 and 90°C. After heating, the samples were cooled in ice water for 30 min.

Physical properties of thermal gel

Thermal gel was cut to 5 cm in height and its breaking strength (g) and breaking deformation (%) were measured by using a creep meter (riasingRHEONER2, Yamaden, Tokyo, Japan). A spherical plunger (5.0 mm in diameter) was used and raising rate of stage was 1 mm/s.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli [7] on a 5-20% polyacrylamide gel (e-PAGEL, ATTO Corporation, Tokyo, Japan). A 0.5 g of the sample was homogenized with 20 mL of 2% 2-mercaptoethanol- 20 mM Tris-HCl (pH 8.8) -2% sodium dodecyl sulfate (SDS) -8M urea. The polyacrylamide gel was stained with 0.1% Coomassie Brilliant Blue R-250 and destained in methanol/ acetic acid/ water (3: 1: 6 v/v/v).

Statistical analysis

Statistical analysis was conducted based on Tukey's test [8] by using Excel Toukei 2010 software (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results and discussion

The thermal gel was not obtained when heating the recovered protein without protease inhibitors and in the presence of EDTA, except that very weak gels could be obtained when heated at 90°C. On the other hand, in the presence of serine protease inhibitors (Benzamidine, SBTI and PMSF), thermal gels were obtained when heated at 40, 60 and 90°C. Moreover, the serine protease inhibitors mix (SPIM, Mixture of 50 mM Benzamidine, 1.0 mg/g SBTI and 5 mM PMSF) significantly enhanced physical properties of thermal gels more than single inhibitors did (Fig. 1). Moreover, it was founded that the degradation of myosin heavy chain (MHC) of thermal gels with SPIM was effectively suppressed in SDS-PAGE pattern (data not shown). There are a lot of study reported that endogenous serine protease affects proteolysis of fish meat [9, 10]. Therefore, one of the dominant endogenous proteases of krill protein, which causes low gel-forming ability of recovered krill protein is serine protease.

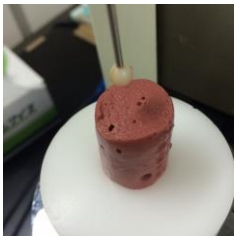

Fig. 1. Thermal gel from recovered North Pacific krill protein in the presence of serine protease inhibitors mix (50 mM Benzamidine, 1.0 mg/g SBTI and 5 mM PMSF) heated at 90°C for 30 min.

Conclusions

Thermal gel-forming ability of recovered North Pacific krill protein using NaCl solution treatment in the presence of protease inhibitors was clarified in the study. By the addition of serine protease inhibitors such as benzamidine, SBTI and PMSF, physical properties of the thermal gels were improved compared to those of control (without protease inhibitors).

These results indicate that recovered North Pacific krill protein can be utilized for gelled product by suppressing endogenous serine protease.

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