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Note

Temperature Effect on Pink Shrimp (*Pandalus eous*) Protein Adsorption onto a Stainless Steel Surface

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To improve the cleaning efficiency of food production equipment, it is crucial to understand the formation of protein deposits. This study examined the adsorption behavior of shrimp (pink shrimp, *Pandalus eous*) proteins onto a stainless steel surface at temperatures of 30 − 95℃, using a shrimp extract solution as a model shrimp food. At temperatures above 60℃, the total amount of adsorbed proteins increased rapidly. However, the adsorption ratio of tropomyosin, a major shrimp allergen, tended to decrease. Results of FT-IR analysis of heated shrimp extract solutions suggested that the intermolecular aggregation, occurring by thermal denaturation of proteins in the extract above 60℃, affected the adsorption behavior of proteins. SDS-PAGE of the extract solutions before and after the adsorption procedure showed that the type of adsorbed proteins onto the stainless steel surface changed markedly at temperatures above 60℃.

Keywords: shrimp protein, tropomyosin, stainless steel surface, adsorption, protein aggregation

Introduction

Adsorption of proteins onto solid surfaces is an important issue in food and drug manufacturing processes (Nakanishi *et al*., 2001). In such processes, protein deposits often form on equipment surfaces. These deposits can act as a nutrient for product spoiling microorganisms and pathogens. In cases where the same equipment is used for the production of different foods, contamination of other food ingredients might occur. Therefore, equipment surfaces must be cleaned on a regular basis to remove any deposits. In general, equipment cleaning requires large amounts of water, detergent, and energy. Understanding the deposit formation process is necessary to improve cleaning efficiency. Because stainless steel has been used extensively as a material for food and drug manufacturing equipment, adsorption behavior onto a stainless steel surface was investigated for food proteins such as bovine β-lactoglobulin (Itoh *et al*., 1995), lysozyme (Sugiyama *et al*., 2012), ovalbumin (Sugiyama *et al*., 2012), and gelatin (Sakiyama *et al*., 1998). Effects of temperature were also reported (Itoh *et al*., 1995; Sakiyama *et al*., 1998). Itoh *et al*. (1995) observed that the amount of β-lactoglobulin adsorbed onto a stainless surface increased steeply above its denaturation temperature. They suggested that this resulted from the thermal aggregation of denatured β-lactoglobulin at the surface; the denatured protein molecules then aggregate to the protein molecules adhering directly to the surface.

Shrimp, which is eaten worldwide, is a major cause of food allergies (Lehrer *et al*., 1992). The main shrimp allergen is tropomyosin, a protein with a molecular weight of about 34 − 39 kDa (Lopata *et al*., 2010). It is important to elucidate the adsorption behavior of shrimp proteins, not only to make the cleaning process more efficient, but also to prevent allergic reactions from shrimp allergen contamination. In a previous study, we investigated the adsorption behavior of proteins in a shrimp extract solution onto a stainless steel surface at 25℃ (Thammathongchat *et al*., 2010). Results suggested that shrimp

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proteins were adsorbed onto the stainless surface, ultimately forming a protein monolayer, which is similar to the food proteins described above (Itoh *et al*., 1995; Sugiyama *et al*., 2012). It was also observed that tropomyosin adhered to a greater degree onto a stainless steel surface than other proteins in the extract.

Shrimp is often heated before eating. Proteins are denatured and sometimes aggregated to each other by heating. The denaturation and the aggregation might affect the shrimp protein adsorption behavior onto a solid surface in a similar manner as for other food proteins (Itoh *et al*., 1995; Sakiyama *et al*., 1998). However, few studies have investigated the adsorption behavior of shrimp proteins with respect to cooking temperature.

The purpose of this study was to investigate the adsorption behavior of shrimp proteins onto a solid surface at > room temperature using a shrimp extract solution as a model shrimp food. Stainless steel was chosen as the solid surface material because it is a material commonly utilized in food processing equipment.

First, the effects of higher temperatures on the amount of adsorbed shrimp proteins, including tropomyosin, were investigated using the depletion method.

Secondly, using Fourier transformed infrared (FT-IR) spectroscopy, alterations in the secondary structure of shrimp proteins by heating were evaluated to assess the correlation between the amount adsorbed at higher temperatures and protein aggregation caused by thermal denaturation.

Finally, SDS-PAGE was conducted to investigate the types of proteins adsorbed at different temperatures.

Materials and Methods

Shrimp sample Pink shrimp (*Pandalus eous*) was used according to a previous study (Thammathongchat *et al*., 2010). Fresh samples were obtained at a wholesale market and stored at −80℃ until used in experiments.

Stainless steel material Fine stainless steel particles of type 316L (3 − 5 μm in diameter, specific surface area 0.58 m^2/g; Epson Atmix Corp., Hachinohe, Japan) were employed in experiments. Before adsorption, the stainless particles were washed using a method similar to that reported previously (Sakiyama *et al*., 1999; Thammathongchat *et al*., 2010). The particles were washed with 0.1 M NaOH at 60℃ for 1 hr with stirring. Particles were rinsed thoroughly with distilled water until the pH of the rinse water was 7.0 and then subsequently dried in an oven at 50℃.

Preparation of shrimp extract solution A similar method to that previously reported (Thammathongchat *et al*., 2010) was used. A frozen shrimp was thawed in running tap water for 20 min. Six grams of edible shrimp meat was mixed with 100 mL distilled water and homogenized using a stomacher (Stomacher 4000 circulator; Seward Ltd., UK) at 230 rpm for 3 min. The resulting homogenate was centrifuged at 4000 rpm and 5℃ for 20 min. The supernatant was then filtered through a 0.45 μm syringe filter (Sartorius AG, Germany) to remove insoluble substances. Total protein concentration in the filtrate was measured using the BCA method (Thermo Scientific Pierce). Then, the total protein concentration was adjusted to 1.5 mg/mL by adding distilled water. This solution was used for subsequent adsorption experiments. The tropomyosin concentration in the solution was determined by sandwich ELISA as previously reported (Thammathongchat *et al*., 2010). The tropomyosin concentration in the solution was 0.8 − 2.4% of total protein and varied according to the extraction lot.

Amount of adsorbed total protein and tropomyosin The adsorption procedure was identical to that previously reported (Thammathongchat *et al*., 2010) and was based on the following depletion method (Itoh *et al*., 1995). Two grams of stainless steel particles were mixed with 1 mL of shrimp extract solution in a 20-mL vial. The vial was tightly sealed and incubated at a constant temperature (30 − 95℃) with vigorous shaking for 2 h.

After incubation, concentrations of total protein and tropomyosin in the supernatant were measured using the BCA method and sandwich ELISA, respectively. The amount of total protein or tropomyosin adsorbed onto a stainless steel surface was determined from the difference between the concentrations before and after incubation, as described previously (Thammathongchat *et al*., 2010).

FT-IR measurement Shrimp extract (1.5 mg/mL total protein concentration) was placed in a vial and heated in a water bath for 2 hr at 30, 40, 50, 60, or 80℃. After cooling at room temperature for 2 hr, the extract was concentrated by ultrafiltration (Amicon Ultra 3K device; Millipore Corp.) to obtain a clear IR spectrum. No visible aggregates or precipitates were observed after ultrafiltration. The concentration ratio was about 10. This concentrated extract was used for the following FT-IR measurement.

A spectrometer (Nicolet 6700 FT-IR; Thermo Fisher Scientific Inc., Waltham, USA) equipped with a diamond ATR accessory and DLaTGS detector was used at room temperature. For each spectrum, a 64-scan interferogram was collected at a resolution of 4 cm^{-1}. A distilled water spectrum was subtracted from that of the concentrated sample solution as a background. The spectrum was processed to correct the effects of ATR geometry and ATR crystal material using software (OMNIC; Thermo Fisher Scientific Inc.).

SDS-PAGE of supernatant The adsorption procedure was performed using an almost identical method as for the measurement of adsorbed amount; however, the weight of stainless steel particles was reduced to 1 − 0.25 g. This was because a preliminary experiment using 2 g stainless steel particles did not result in clear protein bands at higher temperatures, indicating that the amount of proteins remaining in the supernatant was below the detection level.

After the adsorption procedure, the supernatant was subjected to 12.5% SDS-PAGE according to the method of Laemmli. As a control, a shrimp extract solution not subjected to the adsorption procedure was also examined. Following electrophoresis, gels were

stained with Coomassie Brilliant Blue. The stained gel was scanned using a flatbed scanner (GT-X970; Seiko Epson Corp., Suwa, Japan) and images were recorded as digital data. By comparing the band patterns between the supernatant and the control, the types of proteins adsorbed favorably were determined.

Data analysis Each experimental run was replicated at least three times. The Student's *t*-test (to examine two samples assuming an equal variance for each) or analysis of variance (ANOVA) followed by Tukey's multiple comparison test (to examine more than two samples) was carried out. Statistical significance was set at a *p*-value of less than 0.05.

Results

Adsorbed amounts of total protein and tropomyosin Figure 1 is a plot of the amount of adsorbed total protein at different incubation temperatures. The adsorbed amounts increased above 60℃. Figure 2 presents a comparison of the adsorption behavior of total protein and tropomyosin at incubation temperatures of 30, 60, and 95℃. Because the tropomyosin concentration in the solution varied according to the extraction lot and was only 0.8 − 2.4% of the total protein, the adsorbed amounts relative to initial values were used to compare adsorption. The amount of tropomyosin adsorbed (> 90%) was significantly higher than that of total proteins at 30 and 60℃ (40 − 50%). At 95℃, tropomyosin adsorption decreased significantly, showing the same level as total protein. This was opposite to the trend observed for total proteins.

FT-IR Figure 3 shows a typical IR spectrum of the extract heated at different temperatures. Amide I, a specific adsorption peak for proteins that reflects the composition of the secondary structure of proteins (Stuart, 2004), was observed around 1600 − 1700 cm^{-1} for all samples. Samples heated at 80℃ showed a sub-peak at around 1620 cm^{-1}, which is indicative of intermolecular β-sheets of proteins and is associated with thermal protein aggregation (Allain *et al*., 1999; Carbonaro *et al*., 2008; Kato and Takagi, 1988; Militello *et al*., 2004).

SDS-PAGE SDS-PAGE of the extract solutions before and after the adsorption procedure are shown in Fig. 4. The intensity of protein bands in the supernatants tended to decrease after the adsorption process. Therefore, the protein concentration in the extract solution decreased from adhesion of the protein onto the surface of stainless steel particles. The protein bands after incubation tended to disappear gradually by increasing the

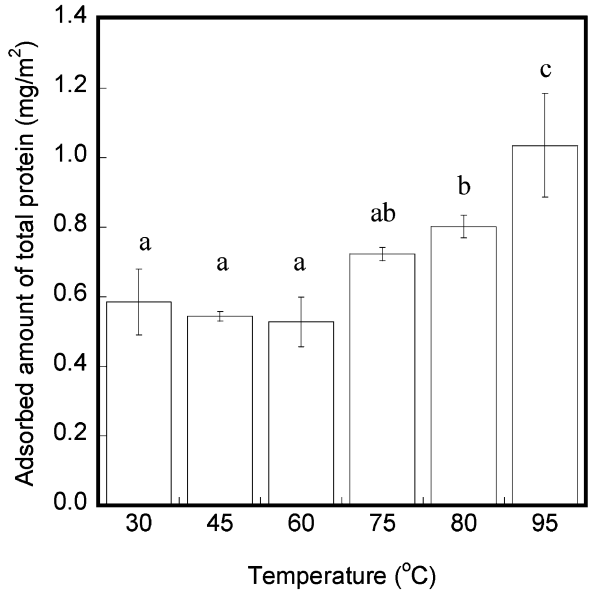

Fig. 1. Plot of adsorbed total protein amounts at different incubation temperatures. Data with the same letter indicates that the groups were not significantly different ($p < 0.05$, Tukey's multiple comparison test).

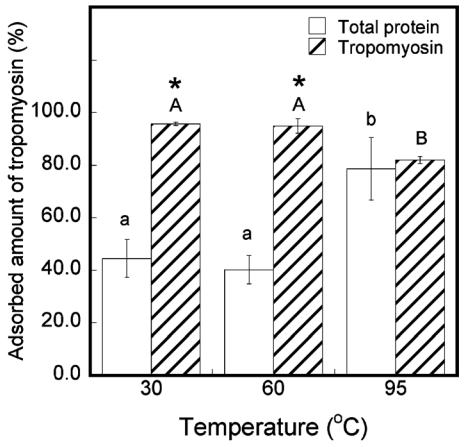

Fig. 2. Total protein and tropomyosin adsorption ratios at incubation temperatures of 30, 60, and 95℃. An asterisk indicates that the value is significantly different from that for total protein ($p < 0.05$, Student's *t*-test). Data with the same lower-case letter among total protein or the same capital letter among tropomyosin indicates that the groups were not significantly different ($p < 0.05$, Tukey's multiple comparison test).

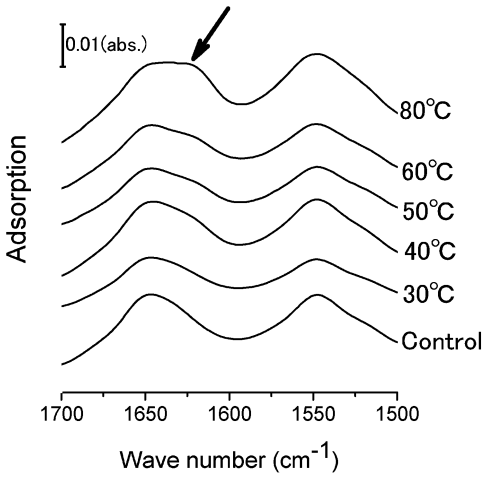

Fig. 3. Typical IR spectrum of extract heated at different temperatures. The IR spectrum of sample heated at 80℃ show a sub-peak at around 1620 cm^{-1} as indicated by the arrow, which is indicative of intermolecular β-sheets of proteins and is associated with thermal protein aggregation.

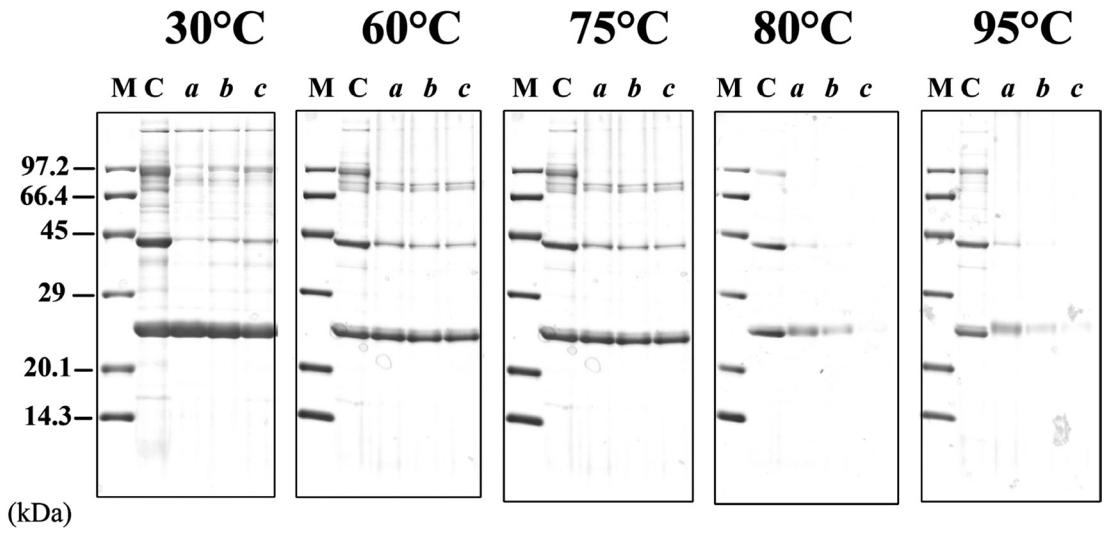

Fig. 4. SDS-PAGE patterns of extract solutions after adsorption procedure at different temperatures. M, molecular weight marker; C, extract solution before adsorption procedure; *a- c*, extract solution after adsorption procedure. The weights of stainless steel particles for *a, b* and *c* were 0.25 g, 0.50 g and 1.0 g, respectively.

incubation temperature from 60 to 95℃, indicating an increase in the amount of adsorbed proteins. This observation agreed with the result in Fig. 1. Protein bands weakened non-uniformly after the adsorption procedure. For example, at 30℃, the 25 kDa protein band did not weaken, but the 45 kDa band almost disappeared. This difference was observed among the samples heated at different temperatures. For example the 25 kDa protein band did not change at 30 and 60℃, but became significantly weaker at 80 and 95℃.

Discussion

The FT-IR result suggested that above 60℃ a large amount of proteins in the shrimp extract aggregated and caused an increase in the amount of proteins adsorbed, as previously reported for β-lactoglobulin (Itoh *et al*., 1995) and bovine serum albumin (Sakiyama *et al*., 1998), even though the shrimp extract was composed of a protein mixture. The decreased adsorption of tropomyosin above 60℃ (Fig. 2) may be also explained by protein aggregation due to heat denaturation. As for shrimp tropomyosin, Uddin *et al*. (2002) reported that tropomyosin from kuruma prawn (*Penaeus japonicus*) was highly thermostable and resistant to heat-induced aggregation up to 108℃, even in the presence of other proteins. Despite differences in shrimp species, the resistance to heat-induced aggregation might be similar for pink shrimp tropomyosin because of the high amino acid sequence identity (96.8%) between kuruma prawn and pink shrimp (Motoyama *et al*., 2007). Therefore, results suggest that at the temperatures examined in this study (30 − 95℃), tropomyosin in the sample solution showed minimal aggregation, although a considerable amount of other proteins accumulated onto the stainless steel surface as a result of heat-induced aggregation. This difference in aggregation behavior might explain the lower relative adsorption favorability of tropomyosin above 60℃. Despite this, tropomyosin was still adsorbed onto the stainless steel particles at temperatures associated with cooking, such as boiling, because the adsorption ratio of tropomyosin at 95℃ was higher than 75%. SDS-PAGE also clearly showed that favorably adsorbed proteins differed according to temperature. This might result from differences in the denaturation temperature or aggregation kinetics among individual proteins.

The shrimp extract contained a mixture of different proteins. Using SDS-PAGE, we obtained information regarding adsorption favorability of specific proteins in the extract as shown above. Protein adsorption has been investigated extensively by many researchers. Most studies, however, have specifically addressed the adsorption of specific individual proteins, even though food ingredients are typically a mixture of different proteins. Therefore, information on the adsorption behavior of proteins in a mixture is necessary to understand protein adsorption in practical situations. SDS-PAGE is a facile method and can be potentially used to investigate the adsorption behavior of proteins in a mixture.

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