Histological and Biochemical Changes of Muscle Collagen during Chilled Storage of the Kuruma Prawn *P. japonicus*

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Histological and biochemical changes of collagen in the kuruma prawn *P. japonicus* muscle during chilled storage were examined. In histological experiments, a partial disintegration of thin connective tissues, endomysium and perimysium, where a specific α component, α2(AR-I), mainly distributed, was observed in the prawn muscle stored for 24 h at 5°C, while the structure of the thick connective tissue, epimysium, did not so changed. On the other hand, a preferential decrease in the relative staining intensity of the components reactive for the anti-α2(AR-I) component serum was detected by immunoblot analysis of guanidine hydrochloride-soluble collagen from the muscle stored for 24 h at 5°C, while the α1(AR-I) component or Type AR-II collagen did not so affected. These combined results suggest that the disintegration of the thin connective tissues is due to some biochemical changes of α2(AR-I)-related components.

Key words: collagen, prawn, muscle, chilled storage, *P. japonicus*, histology, crustacean

A number of investigators have pointed out that prawns are such a perishable food that they should be stored in adequate refrigeration and handled promptly. According to organoleptic studies, ice-stored prawns exhibited three general flavor changes indicating that the palatability and the sweet-flavor characteristic of fresh prawns change to a flat taste, followed by another sharp change in flavor which denotes onset of spoilage. Studies on the various qualitative changes of prawn muscle during storage have been reported by many workers, mainly focusing on the biochemical, organoleptic, and bacteriological methods for estimating the degree of freshness of stored muscles of commercially important Penaeid prawns, especially brown shrimp *P. azteca*, white shrimp *P. setiferus*, or kuruma prawn. Nishimura et al. examined the changes of Sujiebi *Palaemon paucidens* muscle during frozen storage by the measurement of actomyosin Ca²⁺-ATPase activity. Recently, Matsumoto and Yamanaka have reported the changes in content of ATP related compounds, polyamines, volatile basic nitrogen, and lactic acid in the kuruma prawn muscle during storage at 5°C, 0°C, and −1°C, and examined influences of antibiotics, chloramphenicol, on post-mortem biochemical changes in the muscle during storage. Moreover, the effect of "Arai" treatments on the biochemical changes in the kuruma prawn muscle and rigor-mortis of the kuruma prawn muscle have also been examined.

On the other hand, information on changes of collagen in the prawn muscle during storage is quite limited, except for the studies on the changes of collagen content and texture of the cooked meat of the white shrimp and fresh water prawn, *Macrobrachium rosenbergii*. We have demonstrated the existence of two molecular species of collagen, Type AR-I and AR-II collagens, in the kuruma prawn muscle. The major type of collagen (Type AR-I) contains three molecular forms, denoted as α1(AR-I)1α2(AR-I), α1(AR-I)2α2(AR-I), and α1(AR-I)α2(AR-I)2, respectively, where two kinds of α components are referred to as α1(AR-I) and α2(AR-I). The α1(AR-I) and α2(AR-I) components suggested to be also widely distributed in the muscular tissues of other crustacean species. Recently, we have reported that Type AR-I collagen contributes greatly to the development of raw meat texture of sixteen crustacean species. In the previous paper, we also reported that the collagen in the residual fraction after alkali (0.1 N NaOH) extraction (RS-AL) of the kuruma prawn muscle had a quite low solubility not only in acidic solvent but also in hot-water, and suggested that the low solubility of collagen played a positive role in maintaining the firmness and morphology of the cooked meat. Moreover, collagen has also been focused on as a protein which greatly contributes to meat texture of mammalian, fish, and moluscan species. The purpose of the present study was to elucidate histological and biochemical changes of each type of collagen in the raw meat of kuruma prawn during chilled storage and to discuss the contribution of collagen to its textual change.

Materials and Methods

Preparation of Collagens

Live kuruma prawns (average body weight, 40 g) were obtained from Setonaikai Suisan Kahiatsu Co., Ltd. and bred for a few days in the synthetic seawater, Jamarine U (Jamarine, Japan). They were killed by decapitation and divided into four parts: the first was used immediately for analysis and the others were stored in polyethylene bags divided into four parts: the first was used immediately for logical experiments as described later. The prawns in each part were further divided into four groups as follows. The group I was the sample for determination of total collagen content. In this group, three individuals were subjected separately to the following extraction and collagen determination. The groups II, III, and IV were for estimation of solubility of collagen to 4 M guanidine hydrochloride (G/HCl) and 0.5 M acetic acid, and for preparation of pepsin-solubilized collagen (PSC), respectively. In the last three groups, five individuals were subjected together to the respective experiments.

The abdominal part of the prawns were cleaned of the exoskeleton and adhering tissues, and homogenized with a non-bubbling homogenizer (SN-2, Nissei, Japan), and extracted with 0.1 M NaOH as described previously. The residue after the alkali extraction (RS-AL) was washed thoroughly with distilled water. After centrifugation at 10,000 × g for 20 min, the resultant precipitate was re-homogenized with a microhomogenizer (Physcotron NS-310E, Nichi-on, Japan). The RS-ALs of the groups II and III were extracted with 50 mm Tris-HCl, pH 7.5, containing 4 M G/HCl and 0.5 M acetic acid, respectively, by the same method as described previously. After centrifugation at 10,000 × g for 20 min, the supernatants were dialyzed against distilled water overnight and successively against 0.5 M acetic acid containing 2 M NaCl. The resultant precipitates were collected by centrifugation at 10,000 × g for 20 min, desalted by dialysis against distilled water, and lyophilized. The resultant lyophilized materials were used as G/HCl-soluble collagen (GSC) and acid-soluble collagen fractions, respectively. The residue after the G/HCl extraction of the group II was washed thoroughly with distilled water and lyophilized. PSC was prepared by the same method from the RS-AL of the group IV according to the method described previously.

Determination of Collagen

The RS-AL of the group I was lyophilized and one milligram aliquots of the samples were hydrolyzed in 6 N HCl at 130°C for 3.5 h. Hydroxyproline content in the hydrolysate was determined by the method of Woessner Jr. The collagen content was estimated on the basis of the hydroxyproline content (11.8%) in the collagen from the muscle of Panulirus longipes, and expressed as the relative proportion (%) to wet and dry tissues.

Histological Observations

Histological observation by a light microscope was carried out by the same method as in the previous report. Small pieces of the muscular tissue were dissected, fixed in Bouin's solution for 6 h and embedded in paraffin (Parahisto, Nacalai Tesque, Japan). Six micrometer sections were cut with a microtome. The prepared sections were stained with Azan stain and observed with a light microscope, XF-PH-21 (Nikon, Japan).

For histological observation by a transmission electron microscope, small pieces of the muscle was dissected and fixed in 150 mm sodium cacodylate (TAAB Laboratories Equipment LTD, UK), pH 7.5, containing 2% acrolein (Junsei Chemical Co., LTD, Japan), 2% glutaraldehyde (TAAB), and 15% saccharose for 2 h at room temperature. After washing in 150 mm sodium cacodylate, pH 7.5, containing 15% saccharose and 1.65% NaCl (the rinse buffer), samples were post-fixed in 2% osmic acid in 75 mm sodium cacodylate, pH 7.5, containing 7.5% saccharose and 2.2% NaCl for 1 h. Samples were washed twice in the rinse buffer, and dehydrated in a graded series of ethanol (from 60, 70, 80, 90, 95 to 100%). Following this, tissues were rinsed in QY-2 (Methylglycidyl-ether; Nisshin EM Co., LTD, Japan) for 1 h and then in a 1:1 mixture of QY-2 and TAAB 812 resin which was the mixture of epon 812, dodecyl succinic anhydride, melyn acide anhydride, and 2,4,6-Tri(diethylaminoethyl) phenol in a ratio of 48:19:33:2, respectively. And then tissue pieces were transferred to the resin and embedded. Ultrathin sections (gold—silver) were prepared by an ultramicrotome, and counterstained with uranyl acetate and lead citrate. The samples prepared were observed by a transmission electron microscope, JEOL JEM-1200EX (JEOL, Japan).

Estimation of Muscle Firmness

Muscle firmness was measured by injecting a needle (0.6 × 2.6 mm, KB Shokai, Japan) with a Food Rheometer RE-3305 (Yamaden, Japan) for the muscle of kuruma prawn immediately after death, and stored for 6, 12, 24, 72, and 120 h at 5°C. The muscle was cut in a thickness of 10 mm at right angles to the long body axis, and the injecting area was restricted to the shadowed part depicted in Fig. 8A. The injection was performed to a depth of 5 mm at a rate of 5 mm/s perpendicularly to the cross section. As soon as the needle reached to the depth, it was drawn at the same speed. The resistance increased almost linearly with the injection of the needle. The value of the slope (mg/mm) in the injection was defined as penetration resistance, and the muscle firmness was expressed as this value.

Preparation of Antisera

Antisera against Type AR-Ia collagen, Type AR-II collagen, and the α2(AR-I) component were prepared as described previously. These collagens or α components were dissolved in 0.1 M acetic acid or distilled water at a concentration of 1 mg/ml. The solutions were emulsified in equal volumes of Freund's complete adjuvant. The emulsion (0.5 ml) was injected subcutaneously into the back of a rabbit. After 7 and 20 days the rabbits were further immunized with 0.5 ml of the same antigens. The rabbits were boosted in the same way as above at 7 days prior to the collection of blood. Blood was collected from the ear vein and left overnight in a cold room at 5°C. After centrifugation, the resultant supernatant was used as the antiserum preparation.
**Analytical Methods**

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli\(^43\)) using 5 or 7.5% polyacrylamide gels. The sample (5.0 µl) was applied to a sample well and electrophoresed. Gels were stained for protein with Coomassie Brilliant Blue (CBB) R-250 essentially as described by Fairbanks \textit{et al.}\(^44\). The gel was initially stained by soaking in 10% acetic acid containing 0.05% CBB R-250 and 25% 2-propanol for 1 h at room temperature. The first staining solution was then exchanged for 10% acetic acid containing 0.004% CBB R-250 and 10% 2-propanol. After 2 h, the gel was soaked in 10% acetic acid containing 0.002% CBB R-250 for 2 h. Then the background of the gel was extensively destained with 10% acetic acid. The collagen and related peptides were stained metachromatically and non-collagenous proteins were stained orthochromatically.\(^45,46\)

After separation by SDS-PAGE, collagen chains were transferred to nitrocellulose membranes according to the method of Towbin \textit{et al.}\(^47\), and the membranes were immunostained with the indirect method or avidin-biotin complex method. The nitrocellulose membranes were blocked in 2% hen egg ovalbumin in 10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl (PBS; phosphate buffered saline) and then reacted with each antiserum diluted 1:100 ~ 1:1000 in PBS. The goat anti-rabbit IgG conjugated to horseradish peroxidase (Tago Inc., UK) or the biotinylated goat anti-rabbit IgG (Vector Lab., USA) were diluted 1/500 ~ 1/200 in PBS and used as the secondary antibodies. In the case of the experiment by the avidin-bio-

![Image of micrographs](image-url)

**Fig. 1.** Light micrographs of the kuruma prawn muscle immediately after death (A, C, and E) and stored at 5°C for 24 h (B, D, and F), stained with Azan.

Letters Ep, Pe, and En show epimysium, perimysium, and endomysium, respectively. Bars = 50 µm.
tin complex method, the membranes were further reacted with the avidin-biotin complex. The binding of antibodies was visualized using 0.04% (w/v) diaminobenzidine tetrahydrochloride (Nacalai Tesque, Japan), 0.06% 4-chloro-1-naphthol (Bio-Rad, USA), or 0.02% 4-chloro-1-naphthol and 0.06% o-dianisidine (Nacalai Tesque), containing 0.006% H2O2.

Results

Histological Observations

Figure 1 shows the light microscopy of the kuruma prawn muscle. As reported previously, histological observations were conducted to classify the connective tissues of the kuruma prawn muscle into three types, epimysium, perimysium, and endomysium, based on their location in the muscle. The epimysium is the thick connective tissue which forms the outer sheath of muscular tissues, while the perimysium and endomysium are the thin connective tissues which surround the muscle fiber bundles and each muscle fiber, respectively. These thin connective tissues are often indistinguishable to each other as described in the previous report.

In the case of the muscle immediately after killing, muscular cells were tightly connected to each other through endomysium (Fig. 1A and 1C), whereas the muscle fibers were reduced in thickness and separated from the thin connective tissues in the muscle stored for 24 h (Fig. 1B and 1D). Moreover, fragmentation and longitudinal separation of the endomysium or perimysium were observed in places in the muscle stored for 24 h (Fig. 1B and 1D). On the other hand, epimysium somewhat shrunk but not fragmented by 24 h storage (Fig. 1E and 1F). The muscle stored for 72 or 120 h

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Fig. 2. Electron micrographs of the kuruma prawn muscle immediately after death (A, C, and E) and stored at 5°C for 24 h (B, D, and F). Letters Ep, En, and M show epimysium, endomysium, and muscle fiber, respectively. Bars=1 μm.
showed similar images to those of the muscle stored for 24 h (data not shown).

Figure 2 shows the transmission electron microscopic images of connective tissues in the prawn muscle stored for 0 or 24 h at 5°C. As in Fig. 2A, collagen fibers of pericellular connective tissue were observed in order, and the arrangement of collagen fibrils was also detectable in the muscle of immediately after death. In contrast, separation of the collagen fiber from muscle cells (Fig. 2B, 2C, and 2D), and fragmentation of the endomysium (Fig. 2B and 2D) occurred for the muscle stored for 24 h, and the orientation or arrangement of collagen fibrils became quite unclear (Fig. 2B and 2C). Moreover, some of endomysium was longitudinally separated to be about half in thickness (Fig. 2C). On the other hand, epimysium was not so changed by storage for 24 h, showing a clear image of the arrangement of collagen fibrils, except for the separation from the thin connective tissues (Fig. 2E and 2F).

**Chemical Analysis of Collagens**

Table 1 represents the total collagen content and the relative proportion of GSC to the total collagen of the muscles immediately after death (fresh) and stored for 24, 72, and 120 h at 5°C. The collagen recovered in RS-AL was determined to be 0.55–0.59% per wet tissue and 2.3–2.5% per dry tissue, exhibiting no significant change (p > 0.01) in the total collagen. These results suggest that there is little decrease in the total collagen content by some enzymatic activity during storage. In addition, the GSC was 1.8–2.1% per total collagen, showing that the storage for 24–120 h did not bring a pronounced change to the solubility of collagen upon the extraction with 4 M G/HCl. Incidentally, we could obtain only a trace amount of soluble collagen by the extraction with 0.5 M acetic acid irrespective of storage duration adopted.

Figures 3 and 4 show the SDS-PAGE patterns of the PSC and GSC from the fresh or stored muscles, respectively. The PSC exhibited two α components of Type AR-I collagen, α₁(AR-I) and α₂(AR-I), and a main component of Type AR-II collagen, 180 K component, as reported previously. Little change occurred in the pattern of the PSC during storage up to 120 h, and no other lower molecular weight component than α chains was observed.

![Fig. 3. Densitometric tracing of the SDS-PAGE patterns (7.5% gel) of the pepsin-solubilized collagens from the kuruma prawn muscle immediately after death (A) and stored for 24 h (B), 72 h (C), and 120 h (D) at 5°C. Arrows 1, 2, and 3 show the positions of the α₁(AR-I), α₂(AR-I), and 180 K components, respectively.](image-url)

**Table 1.** The content of collagen recovered in RS-AL*, and the solubility in 4 M guanidine hydrochloride of the kuruma prawn muscle immediately after death and stored for 24, 72, and 120 h at 5°C.

<table>
<thead>
<tr>
<th>Storage time (h)</th>
<th>Collagen content</th>
<th>GSC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of wet tissue†</td>
<td>% of dry tissue †</td>
</tr>
<tr>
<td>0</td>
<td>0.55±0.02</td>
<td>2.3±0.12</td>
</tr>
<tr>
<td>24</td>
<td>0.34±0.02</td>
<td>2.3±0.16</td>
</tr>
<tr>
<td>72</td>
<td>0.38±0.03</td>
<td>2.4±0.17</td>
</tr>
<tr>
<td>120</td>
<td>0.39±0.04</td>
<td>2.5±0.21</td>
</tr>
</tbody>
</table>

* RS-AL, residue after alkali extraction.
† Values are expressed as the average±S.D. for three prawns. No significant difference was detected in each column (p > 0.01).
* GSC, guanidine hydrochloride-soluble collagen.
† Values are expressed as the average of three determination.

The GSC from the fresh muscle showed six α chain-sized components, one β chain-sized component, and several higher molecular weight components (Fig. 4). These α sized components were designated as chains a, b, c, d, e, and f, and the β chain-sized component as chain g as was reported in the previous paper. The GSC from the muscle stored for 24 h showed a similar pattern, but the combined proportion of the chains, designated as e and f, to the total peak area of GSC decreased from 11% to 7%, while the proportion of the chain d increased from 8% to 12% by 24 h-storage.

Changes of the components comprising each molecular species in the GSC, however, could not be adequately observed by SDS-PAGE because the chains were poorly separated. Therefore, the collagen chains shown in the ele-
Fig. 4. Densitometric tracing of the SDS-PAGE patterns (5% gel) of the guanidine hydrochloride-soluble collagens from the kuruma prawn muscle immediately after death (A) and stored for 24 h (B), 72 h (C), and 120 h (D) at 5°C.

Letters a, b, c, d, e, f, and g show the positions of the chains a, b, c, d, e, f, and g, respectively.

trophogram of the GSC were transferred to nitrocellulose membrane and immunostained with anti-Type AR-Ia and AR-II collagens, and anti-α2(AR-I) components sera, of which specificity was examined previously. Figure 5 shows the immunoblot analysis of the GSC from the fresh and stored muscles stained with anti-Type AR-Ia collagen serum. The GSC showed the bands of the chains a(AR-Ia), b(AR-Ia), c(AR-Ia), e(AR-Ia), and g(AR-Ia) (Fig. 5A). The GSC from the stored muscles showed similar patterns to that of the GSC from the fresh muscle, although the band of the chains b(AR-Ia) and c(AR-Ia) became slightly fainter by the storage for 120 h (Fig. 5D). The results of immunoblot analysis using the anti-α2(AR-I) component serum was shown in Fig. 6. The anti-α2(AR-I) component serum reacted to the chains d(α2) and f(α2), and to the bands near the position of the chains c(AR-Ia) and c(AR-Ia), designated as c(α2) and e(α2) chains (Fig. 6A). The chains e(α2) and f(α2) almost disappeared after 24 h-storage in the immunoblot analysis (Fig. 6B, 6C, and 6D). Because almost no decrease of the chain e(AR-Ia) by storage was detected in Fig. 5, the decrease of the chain e observed in the SDS-PAGE pattern of the GSC (Fig. 4) appeared to be due to the decrease of the chain e(α2). Intact Type AR-II collagen showed a main component of 290 K, and minor components 270 K, 260 K and 300 K (Fig. 7A). Although the 270 K and 260 K components slightly increased at 24 h-storage, and the 300 K component decreased at 120 h-storage (Fig. 7D), major changes were not detected in the immunoblot analysis.
Post-Mortem Change in Firmness

Post-mortem change in the penetration resistance during chilled storage is shown in Fig. 8B. The value of penetration resistance of the muscle immediately after death was 2280±190 mg/mm, and the value significantly reduced from 0 time to 12 h after death (p<0.01), and then gradually decreased until 24 h to 1660±43 mg/mm. The values at 72 and 120 h after death were almost identical to the value at 24 h (data not shown). These results indicate that stored prawn muscle softens especially during the first 12 h under the chilled condition, as measured by the penetration test.

Discussion

Mills et al. detected the decrease of thermal shrinkage temperature and increase of thermal solubility at 77°C for 70 min of bovine muscle collagen during the first 24 h following slaughter with the greatest amount of change occurring in the first 8 h post mortem. In fish, it has been suggested that the fine collagen fibrils which arise from myocommata progressively deteriorate during ice storage. Sato et al. reported that the solubility of Type V collagen in the muscle of trout Salmo gairdneri increased significantly during chilled storage, while that of Type I collagen did

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Fig. 6. Densitometric tracing of the immunoblot analysis of the guanidine hydrochloride-soluble collagens from the kuruma prawn muscle immediately after death (A) and stored for 24 h (B), 72 h (C), and 120 h (D) at 5°C.

The samples were separated on 5% polyacrylamide gel, transferred to a nitrocellulose membrane, and immunostained with the anti-α2(AR-I) component serum. Arrows 1, 2, 3, and 4 show the positions of the chains c(α2), d(α2), e(α2), and f(α2), respectively. Arrow a shows the relative mobility of the chain a.

Fig. 7. Densitometric tracing of the immunoblot analysis of the guanidine hydrochloride-soluble collagens from the kuruma prawn muscle immediately after death (A) and stored for 24 h (B), 72 h (C), and 120 h (D) at 5°C.

The samples were separated on 5% polyacrylamide gel, transferred to a nitrocellulose membrane, and immunostained with the anti-Type AR-II collagen serum. Arrows 1, 2, 3, and 4 show the positions of the 300 K, 290 K, 270 K, and 260 K components, respectively. Arrow a: the same as in Fig. 6.
Changes of Collagen in Prawn during Chilled Storage

not change, suggesting that degradation of non-helical regions or intermolecular crosslinks occur preferentially in Type V collagen. Ando et al.\(^ {55,56} \) have demonstrated by light and electron microscopy that post-mortem tenderness of rainbow trout muscle is mainly due to the disintegration of collagen fibers in the pericellular connective tissues. Moreover, it was reported that the thermal solubility at 77°C for 60 min of the collagen in the white shrimp muscle increased from 27.97 to 32.04% during storage of muscle for about 16 days in ice.\(^ {22} \) In the present study, it was demonstrated that no significant change occurred in the content of collagen recovered in the RS-AL, and in the SDS-PAGE pattern of the PSC during storage. Moreover, no pronounced change was observed in the solubility of the muscle collagen to 4 M G/HCl and 0.5 M acetic acid extractions during storage. These results suggest that the whole of the collagen in the muscle remains insoluble to the alkali extraction, and that cleavage of the triple-helical region by a collagenase-like enzyme did not occur during storage, at least, until 120 h.

The present study revealed that the chains e(α2) and f(α2) in the GSC markedly decreased during 24 h-storage, while the α1(AR-I) or Type AR-II collagen-related components was not so affected. As reported in the previous paper,\(^ {22} \) the chains e(α2) and f(α2) reacted only to the anti-α2(AR-I) component serum and neither to the anti-Type AR-Ia collagen serum nor to the anti-Type AR-II collagen serum. Moreover, they are both collagenous components because of their metachromasy on SDS-PAGE. These facts suggest that the chains e(α2) and f(α2) are intact forms of the α2(AR-I) component. We previously demonstrated that the α2(AR-I) component mainly distributed in the thin connective tissues, endomysium and perimysium.\(^ {40} \) In histological experiments, a partial disintegration of the thin connective tissues was observed in the prawn muscle stored for 24 h at 5°C, while the structure of the thick connective tissue, epimysium, did not so change. These combined results suggest that disintegration of the structure of the thin connective tissues is due to some biochemical changes of the multiple forms of the α2(AR-I)-related components such as an enzymatic degradation of their telopeptides.

The present experiment on meat texture showed a significant decrease in the penetration resistance within 24 h after death, which corresponded well to the present histological and biochemical changes of collagen. These facts suggest that the histological and biochemical changes of collagen may have some relation to the change of raw meat texture. Namely, the chains e(α2) and f(α2) may have non-helical domains which affect greatly the interaction among collagen fibrils and between collagen fibers and cell surface, and are susceptible to the endogenous proteolytic activity, although their biochemical changes may not bring a significant enhancement to the solubility of collagen. Structural weakening of the pericellular thin connective tissues, probably due to the degradation of the non-helical domains, may contribute partially to the textural change of the prawn muscle during chilled storage.

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