Improvement of the Material Property of Shark Type I Collagen by Composing with Pig Type I Collagen

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Fibril reconstruction process, that is, the nucleation and growth of mixed type I collagen fibril of shark and pig, progressed faster than that of the individual collagen species of shark or pig. The reconstructed mixed collagen fibril had a greater resistance to return to the solution or to melt into gelatin in comparison with the counterpart consisting solely of shark collagen. The denaturation temperature of the mixed collagen gel was about 10 °C higher than that of shark, and about 5 °C lower than that of pig. By scanning electron microscopy, the diameter of mixed collagen fibril showed an intermediate range between shark and pig collagen fibril. The breaking strength of the mixed collagen gel was tougher than that of pig, but weaker than that of shark. Other physicochemical properties of the mixed type I collagen gel were observed to be at intermediate positions between those of shark and pig type I collagen gels.

Keywords: *Type I collagen; mixed gel from shark and pig collagen; fibrilogenesis; mechanical strength; differential scanning calorimetry*

INTRODUCTION

The molecular structure and biological functions of type I collagen from land animals have been extensively investigated (Kielty et al., 1993; Piez, 1988). Type I collagen, especially bovine skin collagen, has been utilized in foods, cosmetics, and medical materials (Reich, 1995a,b). In recent reports, Sakaguti et al. (1998) showed that collagen peptide can be a food allergen. As an approach to develop new collagen materials that have a modified allergic property, it is considered that aquatic animal collagens are bright prospects for collagen sources and are present in abundance. For type I collagen from aquatic animals, however, there are a limited number of studies, mainly in comparative biochemistry (Kimura et al., 1981a,b; Kimura and Ohno, 1987; Kelly et al., 1988). A few papers have suggested a potentiality of fish gelatins for food and photographic uses (Berg et al., 1985; Leuenberger, 1991). Type I collagen from aquatic animals may provide an alternative collagen source, since its unique features include containing fewer imino acid residues and having a lower denaturation temperature than collagen from land vertebrates (Kimura and Ohno, 1987; Piez, 1988; Kelly et al., 1988; Nomura et al., 1995). Among aquatic type I collagens, shark collagen is potentially important, because an abundant amount of shark is collected in connection with tuna fishery, and the meat and a part of the skin serve as food in Japan. With this connection, our previous paper has shown that shark type I collagen has basically the same ability of self-assembly to form fibril as that of pig type I collagen. However, there must be some differences in the detailed molecular structure between both collagens, and furthermore shark type I collagen forms the fibril under a condition different from that of pig type I collagen (Nomura et al. 1995, 1996, 1997). Such differences in fibrilogenesis must be reflected on the physicochemical property of collagen gel and membrane. In fact, the authors demonstrated that shark collagen gel and membrane had a stronger rigidity and a higher affinity to water vapor than those of pig collagen and hence suggested a potentiality in utilizing shark collagen as a new type I collagen material applicable to various uses, including the cell culture and medical technology (Nomura et al., 2000). However shark collagen has a disadvantage of lower thermal stability in the practical application. Therefore the authors tried to overcome the disadvantage by blending shark collagen with pig collagen. That is, we examined the physicochemical properties of a gel formed from a mixture of shark and pig collagens compared with that from the single species of collagen.

MATERIALS AND METHODS

Purification of Type I Collagen. Type I collagen preparations were obtained from fresh skin corium of great blue shark (*Prionace glauca*) and pig as described in our previous papers (Nomura et al., 1989, 1995).

Collagen Self-Assembly. The self-assembly experiment of collagen was done as described previously (Nomura et al., 1989). Collagen was dissolved in 0.5 M acetic acid at 4 °C and dialyzed against 67 mM phosphate buffer at pH 6.0 or against 67 mM phosphate buffer at a specified pH (5.5 or 6.5). After adjusting the collagen concentration at 0.5 mg/mL, one part of the shark collagen solution (at 0.25 mg/mL) was mixed with one part of the pig collagen solution (at 0.25 mg/mL) and then warmed at 25 °C. This temperature was adopted as the upper limit to avoid the gelatinization of shark collagen during the self-assembly experiment. The collagen self-assembly process was monitored by the absorbance at 310 nm using the UV-spectrophotometer (UV-2000, Shimadzu, Tokyo), as the turbidity change.

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After 24 h, the amount of reconstructed collagen fibril was estimated from the protein concentration of the supernatant after centrifugation at 18000 rpm (29000*g*) for 30 min by the microbiuret method (Itzuhaki and Gill, 1964). The rate of reconstructed collagen fibril was defined as the percentage of the decrease in collagen concentration in the supernatant.

Thermal Behavior of Reconstructed Collagen Gel. *Cooling Stability at 10 °C.* It is known that collagen fibril formation occurs at a higher temperature and its redissolution without gelatinization occurs at a lower temperature and the reaction is substantially reversible. Hence, the redissolution of reconstructed collagen fibril when it is cooled to a low temperature is taken as a feature in thermal behavior (low-temperature stability) of the gel for the concerned collagen species. In the present experiment, a collagen fibril aggregate (gel) formed at 25 °C as described above was cooled quickly to 10 °C, and subsequently the collagen proportion resistant to the dissolution was estimated from the supernatant after centrifugation (at 29000*g* for 30 min) by microbiuret analysis (Itzuhaki and Gill, 1964) in the time course from the cooling to 10 °C.

Heating Solubility at 37 °C. In many biological applications of collagen gel, it is exposed to the temperature circumstance at 35 or 37 °C. Therefore the stability of collagen gel at 37 °C is an important requirement for many uses. In the present experiment, a collagen gel formed at 25 °C as described above was heated quickly to 37 °C, and subsequently the collagen proportion resistant to the dissolution was estimated from the supernatant after centrifugation (by centrifuged at 29000*g* for 30 min) by microbiuret analysis (Itzuhaki and Gill, 1964) in the time course from the heating to 37 °C.

Differential Scanning Calorimetry. The denaturation temperature of the reconstructed collagen fibril was measured by differential scanning calorimetry (DSC). DSC was carried out on a DSC apparatus (Seiko, SSC5000, Tokyo) coupled with a thermal data analysis system DSC 100 as described previously (Nomura et al., 1989). Collagen gel was compressed to a pellet by centrifugation at 3000 rpm (800*g*) for 30 min and washed thrice with 67 mM phosphate buffer at pH 6.0. A portion of the pellet (about 13 mg wet weight) was placed in a Ag cell (75 μL) and subjected to DSC.

Electron Microscopic Observation. The reconstructed collagen fibril was observed under high-resolution scanning electron microscopy (SEM; JSM-6000F, JEOL, Tokyo), as described previously (Nomura et al., 2000).

The specimen for SEM was fixed with 2% glutaraldehyde and dehydrated with alcohol, dried at the critical point at carbon dioxide, and finally coated with gold by ion sputter (JVC-5000, JEOL, Tokyo). An SEM apparatus was operated at 3.0 kV and a magnification of 10000 or 30000.

Mechanical Strength of Collagen Gel. The mechanical strength of the collagen gel (at 0.05−0.15% collagen concentration) was measured by a creep meter (RE-33005, Yamaden, Tokyo), as described previously (Nomura et al., 2000). A 1 mL portion of collagen solution with a concentration of 1 mg/mL was warmed at 25 °C for 24 h on a tissue culture plate (48 well, Becton Dickinson and Co., Franklin Lakes, NJ) to set a gel. Measurement of the breaking strength at the formed gel was done in 60% humidity at 25 °C using a cylindrical probe of 5 mm o.d. moving against the gel at a speed of 5 mm/min. The penetration of the probe was stopped halfway into the whole thickness of the gel specimen. The first peak top or plateau point of the stress−strain curve was defined as the breaking point of a gel specimen.

RESULTS

The Process of Collagen Self-Assembly. In vitro, type I collagen is able to self-assemble spontaneously to form fibril under physiological conditions (Veis and George, 1994). During the incubation at 25 °C, collagen self-assembly was monitored by the absorbance change at 310 nm (Figure 1). The previous study showed that shark collagen had a shorter time of nucleation and longer time of growth for collagen fibrilogenesis than those of pig collagen (Nomura et al., 1997). In the present study, mixed collagen showed a rapid rise in absorbance compared with shark or pig collagen. Thus mixed collagen attained about 70% of the whole increase in absorbance within 15 min of incubation at 25 °C; meanwhile the single collagen of shark or pig did less than 5%. Such a strongly accelerated fibrilogenesis in mixed collagen was observed especially at pH 6.0 and 6.5 (Figure 2), whereas shark collagen did not form fibril entirely at pH 5.5. Even at pH 5.5, the mixed collagen

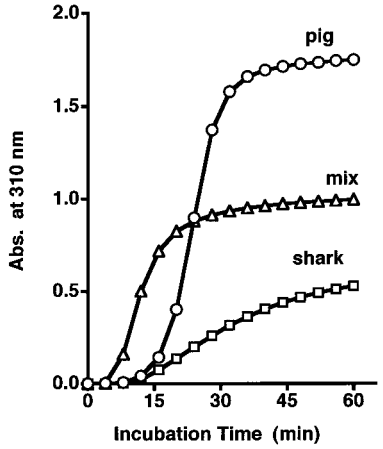

Figure 1. Progress of type I collagen self-assembly from 0.5 mg/mL shark, pig, and mixed collagen solutions (pH 6.0) at 25 °C.

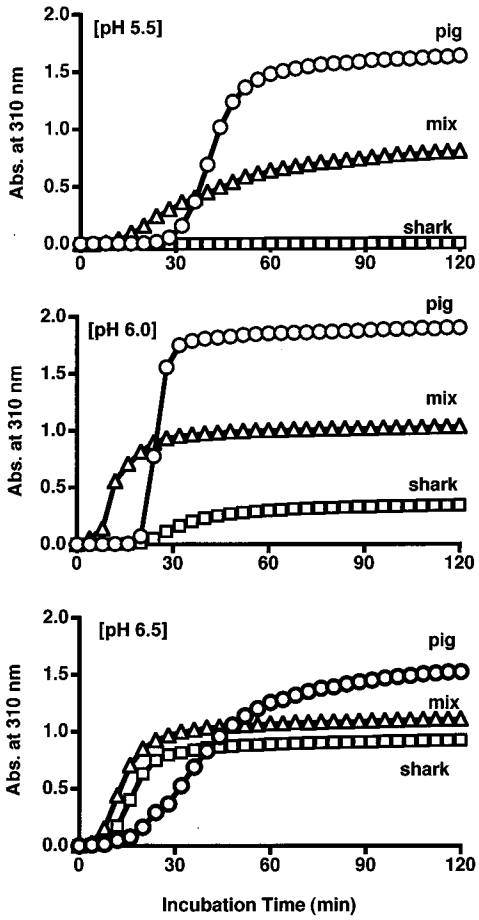

Figure 2. Effect of pH on the progress of type I collagen self-assembly from 0.5 mg/mL shark, pig, and mixed collagen solutions (pH 6.0) at 25 °C.

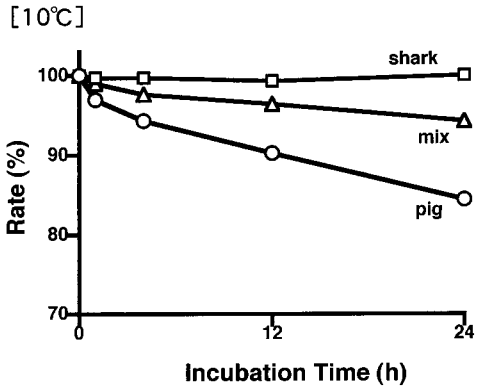

Figure 3. Cooling stability at 10 °C of reconstructed collagen fibrils of shark, pig, and mixed. Collagen (0.5 mg/mL) was reconstructed in phosphate buffer (pH 6.0) at 25 °C for 24 h.

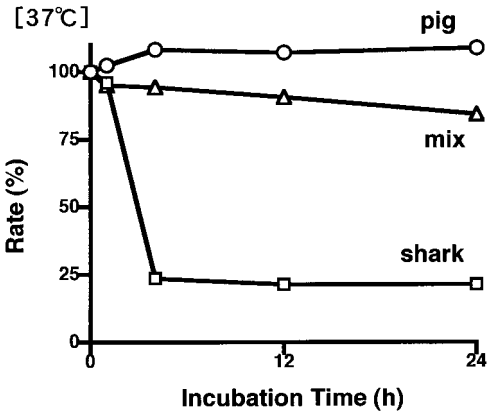

Figure 4. Heating solubility at 37 °C of reconstructed collagen fibrils of shark, pig, and mixed. Collagen (0.5 mg/mL) was reconstructed in phosphate buffer (pH 6.0) at 25 °C for 24 h.

indicated a certain increase in the rate of absorbance increment. These results suggest some interaction occurred between shark collagen and pig collagen.

Cooling Stability of the Reconstructed Collagen Gel. Figure 3 shows plots of the collagen proportion that remained as fibril against the 10 °C incubation time of the gel formed at 25 °C for 24 h. While the pig collagen gel was redissolved by 20%, the shark collagen gel was not redissolved substantially. It is considered that some additional intermolecular interaction that has not been known for pig or land animal collagen species takes place in shark collagen after the fibril has formed. Furthermore, it is more noticeable that mixed collagen fibril demonstrated almost the same resistance to cooling as that of shark single collagen fibril. This suggests that some additional interaction takes place between pig and shark collagens after the fibril has formed.

Thermal Stability of the Reconstructed Collagen Gel. Figure 4 shows plots of the collagen proportion that remained as fibril against the 37 °C incubation time of the gel formed at 25 °C for 24 h. The shark collagen gel quickly dissoluted into solution to about 25% of the starting level within 4 h. Although a part of the shark collagen gel denatured and melted into gelatin, the residual part of the collagen remained undissolved after that, suggesting that a certain interaction among fibrils took place during the incubation at 37 °C. However, mixed collagen gel resisted substantially to the incubation at 37 °C (only about 15% of the collagen dissolved into the solution after 24 h). These results suggest that the gelatinization of shark collagen

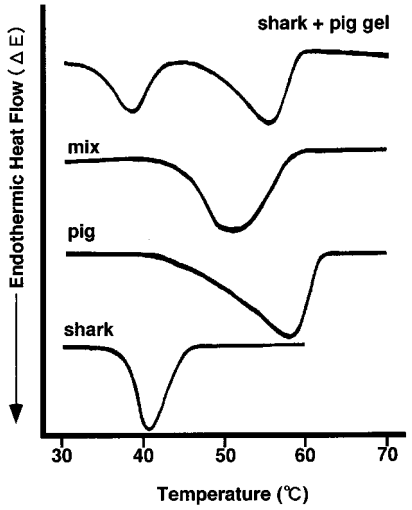

Figure 5. Differential scanning calorimetry curves of collagen gels from shark, pig and mixed. Collagen (0.5 mg/mL) was reconstructed in phosphate buffer (pH 6.0) at 25 °C for 24 h.

is depressed by pig collagen in the mixed gel, and hence a strong interaction between shark and pig collagen is present in the mixed collagen gel.

Thermal Behavior of the Reconstructed Collagen Gel. DSC curves of reconstructed collagen gel are shown in Figure 5. The DSC curve of the shark collagen gel indicated a peak of endotherm around 41 °C and that of pig collagen indicated a peak around 58 °C. The DSC curve from mixed collagen gel indicated a somewhat broad peak shifted to a higher temperature (about 51 °C) compared with that from shark collagen gel. The individuality of both collagens in gelatinization temperature was not affected by the coexistence or mixture of both gels in the same DSC cell. However, when both collagens were mixed in the solution and then formed into the gel (mixed gel), its DSC curve indicated surprisingly only one peak around 51 °C. This means that helical collagen molecules of shark and pig in the mixed gel collapse as a unity into random coils. In other words, it means that pig collagen in the mixed gel depresses shark collagen from gelatinizing at 41 °C and raises the gelatinization temperature of the latter to 51 °C.

SEM Observation of the Mixed Collagen Gel. SEM examination of mixed, shark, and pig collagen gels demonstrated a well-developed fibril network of a mean diameter at about 40−150 nm (Figure 6). The diameter of the mixed collagen was the thinnest, indicating the main value at 46 nm. Some extraordinarily thin fibrils were observed in the mixed and the pig collagen, but not in the shark collagen. The morphology of the mixed collagen fibril resembled that of the pig collagen fibril.

Mechanical Strength of the Reconstructed Collagen Gel. With the increasing collagen concentration, the mechanical strength of the mixed collagen gel rose at a rapid slope equivalent to that of the shark collagen gel, whereas the strength of the pig collagen remained a slow slope (Figure 7). It is noticeable that the strength of the mixed collagen gel stands at a position close to that of the shark collagen gel rather than at an intermediate position between shark and pig.

DISCUSSION

The properties of the mixed collagen gel did not show an intermediate of individual collagens; that is, the

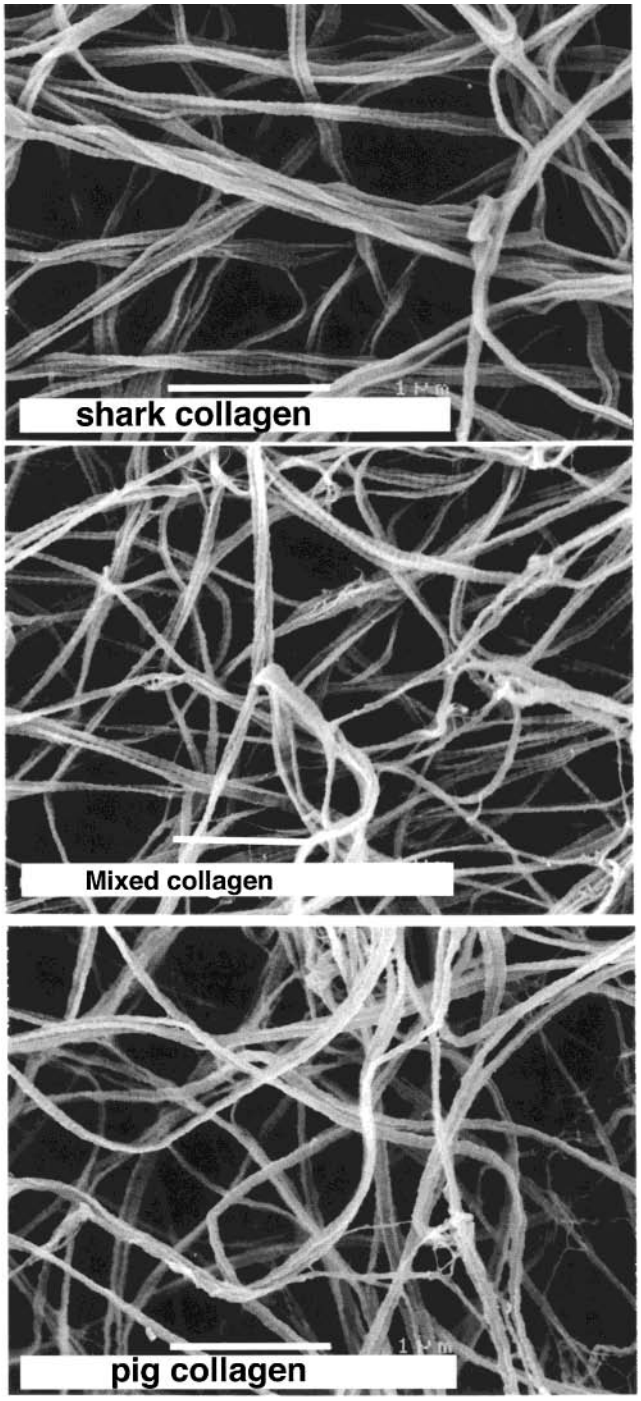

Figure 6. Scanning electron micrographs of collagen fibrils of shark, pig, and mixed. Collagen (0.5 mg/mL) was reconstructed in phosphate buffer (pH 6.0) at 25 °C. Magnifications: 35 000× (reproduced at 70% of its original size). Bars represent 1 μm.

mechanical strength and low-temperature stability of the gel resembled closely that of shark collagen, and the thermal stability of the gel resembled that of pig collagen (Figures 3, 4, and 7). The rate of fibril formation of the mixed collagen was far faster than that of pig or shark (Figures 1 and 2). Probably the production of pig and shark mixed collagen oligomers (nucleus for fibril reconstruction) accelerates the overall rate of fibril reconstruction, as shown by a shortened lag phase of fibril reconstruction in Figure 1. The DSC curve of the mixed collagen gel demonstrated a monomodal peak at about 51 °C rather than bimodal peaks as observed in

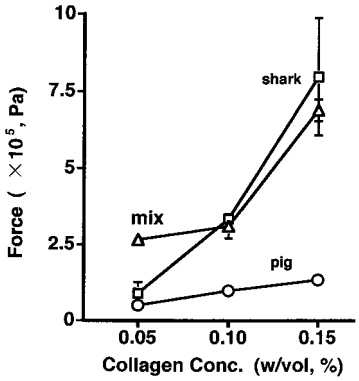

Figure 7. Mechanical strength of reconstructed collagen gels (0.05~0.15 mg/mL) of shark, pig, and mixed in phosphate buffer (pH 6.0) at 25 °C. Bars represent standard deviation (n = 6).

the case of the post-fibrilogenesis blending of shark and pig collagen gels (Figure 5). These results suggest that a strong and specific interaction is present between shark and pig collagens in the mixed gel. There has been no report to suggest the possibility of such a strong interaction among type I collagens from different sources. Type I collagens are widely distributed in the body and binds a minor component of collagen (FACIT collagen), having a distinct physiological property (Birk et al., 1991). Birk et al. (1990) showed that the mixed collagen of types I and V reconstructed thinner fibrils than type I collagen itself. Romanic et al. (1991) reported that copolymerization of pN collagen type III and collagen type I generated fibrils that were thinner than those from collagen type I alone generated under the same conditions. Therefore it is likely that the copolymerization of shark and pig collagens can proceed in such a way that both collagen molecules compose a common network of fibril or a composite fibril network. The present SEM examination suggested that the mixed collagen fibrils had a relative heterogeneity in fibril diameter in comparison with a relative homogeneity for shark collagen fibrils (Figure 6). It is considered that mixed collagen fibrils composed of shark to shark collagen molecules, pig to pig collagen molecules, and shark to pig collagen molecules utilize ionic bound, hydrogen bound, and hydrophbic interactions.

The most important feature of aquatic animal collagens is a denaturation temperature lower than that of land animal collagens. It provides for utilization, for example, in cold food materials, such as to increase viscosity, to make gelation, and to hold water. Such a coating gel needs a certain stability to heat denaturation and a proper mechanical property. However, the present concept of composite collagen gels of shark and another land animal offers a promising plan to overcome the disadvantage of aquatic collagen and to expand its utilization.

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