The Physicochemical Property of Shark Type I Collagen Gel and Membrane

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The physicochemical properties of shark type I collagen gel and membrane were not same as those of pig type I collagen. The denaturation temperature of shark collagen gel was about 15 °C lower. According to scanning electronic micrography, the diameter of shark collagen fibril was relatively thin and more homogeneous. The breaking strength of shark collagen gel was greater, and shark collagen membrane had a greater mechanical strength and a higher water vapor sorption.

Keywords: *Collagen; shark; light scattering; mechanical strength; differential scanning calorimetry*

INTRODUCTION

The molecular structure and biological functions of type I collagen from land animals have been investigated extensively (Kielty et al., 1993; Piez et al., 1988). Type I collagen, especially bovine skin collagen, has been used in foods, cosmetics, and medical materials (Reich, 1995a,b). For type I collagen from aquatic animals, however, a limited number of studies have been published, mainly in comparative biochemistry (Kimura et al., 1981a,b; Kimura and Ohno, 1987; Kelly et al., 1988). A few articles have suggested the possibility of fish gelatins for food and photographic uses (Berg et al., 1985; Leuenberger et al., 1991). Type I collagen from aquatic animals may provide an alternative collagen source, because it contains fewer imino acid residues and has a lower denaturation temperature than collagen from land vertebrates (Kimura and Ohno, 1987; Piez et al., 1988; Kelly et al., 1988; Nomura et al., 1995). More information needs to be collected on the structural features and the industrial potentiality of aquatic animal collagen. Among aquatic type I collagens, shark collagen is potentially important, because abundant amounts of shark are collected in connection with tuna fishery, and the meat and a part of the skin serve as food in Japan. Therefore, we inquired about the properties of shark collagen gel or membrane in this article, because collagen gel provides promising material for various uses including cell culture and medical technology. In this connection, our previous article showed that shark type I collagen has basically the same ability of self-assembly to form fibrils as that of pig type I collagen. However, the details must differ between the two collagens, because both collagens have their own features in molecular structure, and shark type I collagen forms fibrils under conditions different from those of pig type I collagen fibrilogenesis (Nomura et al., 1995, 1996, 1997). There are some reports of physicochemical studies of collagen gel. Danielsen (1990) showed the age-related thermal stability of rat bone and skin by the area shrinkage without tension during gradual heating, and the age-related decrease in thermal stability of bone collagen. Rheological studies of injectable collagen biomaterials (Rosenblatt et al., 1993) and reconstituted type I collagen in confined compression (Knapp et al., 1997) showed that the hydrophobic forces in injectable solvent mediated the dispersion rheological behavior through changes in fiber rigidities, and the fibrils under compression yielded a smaller modulus. However, no report has compared the rheological difference of collagen from different sources. In this report, the physicochemical property of the gel or membrane from shark type I collagen was compared with that of pig type I collagen.

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MATERIALS AND METHODS

Purification of Type I Collagen. Type I collagen was prepared from fresh skin of the great blue shark (*Prionace glauca*) and pig skin as described in our previous articles (Nomura et al., 1989, 1995).

Formation of Gel and Membrane from Collagen Solution. Formation of collagen gel was done as follows: Collagen was dissolved in 0.01 M acetic acid at 4 °C (about 1.0 mg/mL). After 18 h, the solution was centrifuged at 35 000 rpm (100 000*g*) for 1 h (SCP85H, Hitachi, Tokyo), and the clarified solution was dialyzed against M/15 phosphate buffer (pH 6) at 4 °C. After adjusting the collagen concentration from 0.25 to 1.0 mg/mL, the collagen solution was set by warming to 25 °C (Nomura et al., 1994) to form a gel.

The collagen solution (2 mg/mL) was set in minislab gel plate (120 × 102 × 1 mm; AE−6401, ATTO, Tokyo), by warming at 25 °C. The membrane was desalted and dehydrated by sequential soaking in ethanol solutions with stepwise increased concentration to 70, 90, and 99.5%. Such ethanol dehydration procedures are required to desalt and to minimize the deformation of dehydrated membrane. The dehydrated membrane was finally dried by spreading on a mesh.

Collagen concentration was determined by microbiuret method (Itzahki and Gill, 1964).

Differential Scanning Calorimetry (DSC) of Reconstructed Collagen Gel. The denaturation temperature of the collagen gel and membrane was measured by DSC. DSC was performed on a DSC apparatus (Seiko, SSC5000, Tokyo) coupled with a thermal data analysis system DSC 100 as described previously (Nomura, 1989). Collagen gel was compressed to a pellet by centrifugation at 3 000 rpm (800*g*) for 30 min and washed thrice with M/15 phosphate buffer at pH

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6.0. A portion of the pellet (about 13 mg wet weight) was placed in Ag-cell (75 μL) to be subjected to DSC.

Measurement of Dynamic Light Scattering (DLS). DLS (DLS-7000, Ootuka Electronic, Tokyo) analysis was applied to collagen gel to characterize the network structure of shark and pig type I collagen. The scattered incident light from He−Ne laser of wavelength at 632.8 nm was acquired as a function of time at a scattering angle of 90° and at 25° with a selected photomultiplier tube operated in a photon-counting mode. The correlator mode was selected to use time interval methods (T.I.; sampling time, 4 μs; correlator channel, 512). This mode is able to demonstrate the correlation in a relatively short time and gives a diffusion coefficient (D) calculated from the slope of the correlation function to time that is regarded as providing information on the scattering particle size ($D \propto 1/d$; D, diffusion coefficient; d, diameter) and hence is translatable to information on the lattice size of collagen gel in this article.

Electron Microscopic Observation. The collagen gel was observed under a high-resolution scanning electron microscope (SEM; JSM-6000F, JEOL, Tokyo). The SEM specimen was fixed with 2% glutaraldehyde, dehydrated with alcohol, dried by critical point drying with carbon dioxide, and coated with gold by ion sputter (JVC-5000, JEOL, Tokyo). The electron accelerator was operated at 3 kV, and observation was performed at a magnification of 15 000 times.

Mechanical Strength of Gel and Membrane. Rheological strength of collagen gel and membrane was measured by a creep meter (RE−33005, Yamaden, Tokyo). Collagen gel specimen with a concentration from 0.5 to 2 mg/mL was set in tissue culture plate (48 well, Becton Dickinson and Co.), at 25 °C for 24 h. The measurement of breaking strength was done in 60% humidity at 25 °C using a cylindrical probe of 5 mmϕ moving into the gel at a speed of 5 mm/min. The penetration of the probe was stopped at halfway in the whole thickness of the gel specimen. The first peak top or plateau point of stress−strain curve was defined as the breaking point of a gel specimen. Collagen membrane was kept in a desiccator of a standard humidity for longer than 2 days, and was cut to a piece of 5 × 50 mm. Tensile strength of collagen membrane was measured at a stretching speed of 5 mm/min. The peak top point of the stress−strain curve was defined as the breaking point of a membrane specimen.

Water Vapor Sorption of Collagen Membrane. Water vapor sorption of collagen membrane was measured as the weight change of a specimen in a varying moisture condition. Moisture condition was adjusted in a desiccator containing a saturated salt solution to 15% relative humidity (RH) using lithium chloride solution, to 35% RH using chromic trioxide (VI), to 45% RH using potassium nitrite, to 65% RH using magnesium acetate, to 81% RH using ammonium sulfate, to 90% RH using zinc sulfate, and to 98% RH using lead (II) nitrate for 24 h (purchased from Wako, Osaka, Japan). This time span was enough for the film to get to a near equilibrium because the film was very thin (about 0.3 mg/cm^2).

RESULTS AND DISCUSSION

Thermal Denaturation of Collagen Gel. DSC curves of gels from shark and pig collagen were shown in Figure 1. DSC curve from shark collagen indicated only one peak of endotherm, and onset and peak top denaturation (or melting) temperature was estimated as about 39 and 41 °C, respectively, a range of about 3 °C. This denaturation temperature of collagen gel is higher than that of the collagen solution (about 31 °C), which suggests that collagen molecules are more stabilized in the gel network than in solution. The DSC curve of pig collagen gel indicates a somewhat broader band in a higher temperature region (about 58 °C) as compared with that of shark collagen. The sharp and narrow peak for shark collagen gel suggests that a simple endotherm reaction occurred and hence that shark collagen gel is a gel of homogeneous thermal stability.

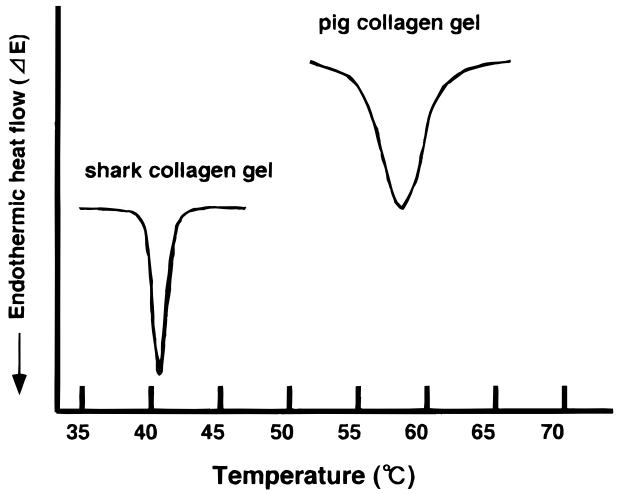

Figure 1. Differential scanning calorimetry curves of collagen gels from shark and pig.

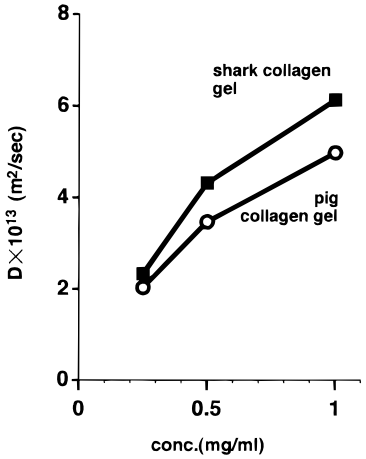

Figure 2. Diffusion coefficient of collagen gels from shark and pig with dynamic light scattering.

The collagen-specific triple helical structure of shark type I collagen is common to that of land mammalian type I collagen, except for a lower concentration of imino acid residue and the presence of intra- or intermolecular cross-linkage from that of land animal collagen (Kimura et al., 1981; Nomura et al., 1995, 1997). Thermal stability of collagen fibril was influenced by the contents of imino acid residues, and collagen triple helix was stabilized with imino acid at the X position of specific sequence Gly−X−Y (Piez et al., 1988). Such a reduced concentration of imino acid residues in shark collagen must contribute to the reduced thermal stability of shark collagen gel, because Burjanadze and Kisiriya (1982) showed experimentally that the denaturation temperature of collagen decreases with decreasing content of hydroxyproline in collagen molecule.

Evaluation of Collagen Gel Structure by DLS and SEM. The characterization of collagen network was done by DLS (Figure 2). Because the D value as determined by DLS of polymer gel is generally proportional to the elastic modulus and hence the polymer concentration of the gel (Tanaka, 1985), the present data as indicated in Figure 2 must reflect this. In this article a particle means a space or solvent volume enclosed by cross-linked polymer chains in gel according to the theoretical treatment of tridimensionally cross-linked polymer gel (Oikawa and Nakanishi, 1993). Hence the

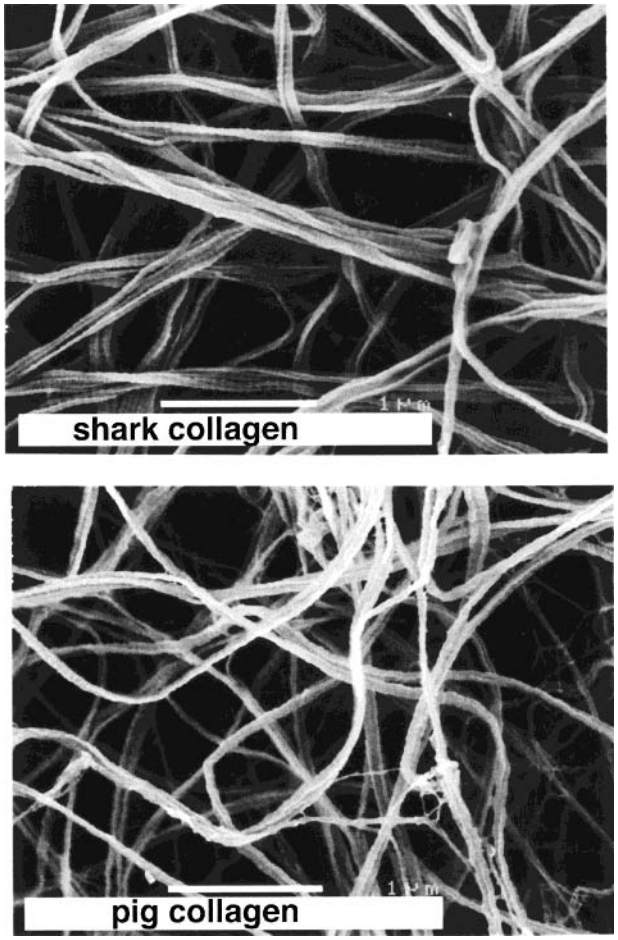

Figure 3. Scanning electron micrographs of collagen fibrils from shark and pig. Magnifications, 35 000×; bars, 1 μm

particle size here means the cell size of the gel lattice. The D value on the order of 10^{-9} cm^2/s of collagen gels corresponds to about 10 000 nm. Hence, it cannot reflect the fibril diameter (collagen fibril diameter is usually about 100 nm), but it can reflect the size of collagen gel cells. The increase in D with collagen concentration may suggest the increase in the number of cluster points (points of interaction between aggregates or fibrils of collagen which includes collectively those of hydrogen bonding, electrostatic, hydrophobic natures, etc.) per volume. Oikawa and Nakanishi (1993) reported that, when a glutaralde-fixed gelatin gel was measured by DLS, the increase in cross-linked points by glutaraldehyde resulted in the limited movement of gelatin gel; hence, the D presented an index for the mobility of cross-linked point of the glutaraldehyde-fixed gelatin gel. Therefore, the increase in D with collagen concentration in the present study reflects the increase in the number of cluster points and hence the reduction in the mobility of clusters of collagen fibril network. The D of shark collagen was larger than that of pig collagen; the shark collagen gel has more cluster points than pig collagen gel.

SEM observation of shark collagen gel demonstrated a well-developed fibril network with a mean diameter at about 87 nm (Figure 3). Its SEM pattern also indicates somewhat thinner, more uniformly and more densely interwoven fibrils than pig collagen gel.

From the results of DLS and SEM, it is suggested that shark collagen gel has a more homogeneously clustered network of collagen fibrils than that of pig collagen gel.

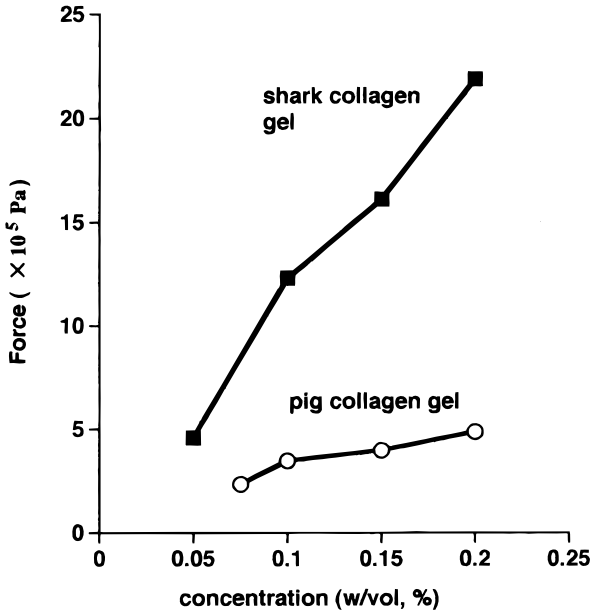

Figure 4. Mechanical strength of collagen gels from shark and pig.

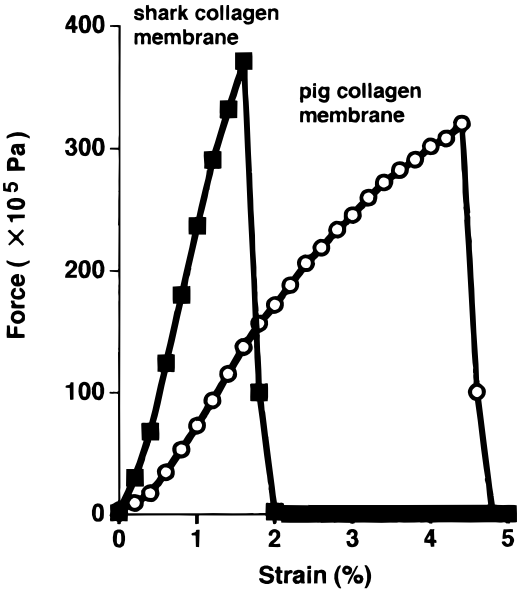

Figure 5. Breaking strength curves of collagen membrane from shark and pig.

Mechanical Strength of Gel and Membrane. Figure 4 shows the effect of collagen concentration on the breaking stress of collagen gel. With increasing collagen concentration, the breaking stress of pig collagen gel indicated only a small rise. However, the breaking stress of shark collagen gel indicated a strikingly rapid rise with increasing collagen concentration. The breaking point of shark and pig collagen gel was estimated as the first peak top or plateau point of stress−strain curves, respectively (data not shown). The result indicates that shark collagen gel is harder than pig collagen gel.

Stress−strain curves of collagen membrane at 0.2% concentration are shown in Figure 5. The curve of shark collagen membrane indicated a rapid rise up to 1.8% of strain and a sudden decline. The curve of pig collagen membrane indicated a similar but far slower rise up to 5% of strain. The breaking stress of shark collagen membrane exhibited a larger value with a shorter

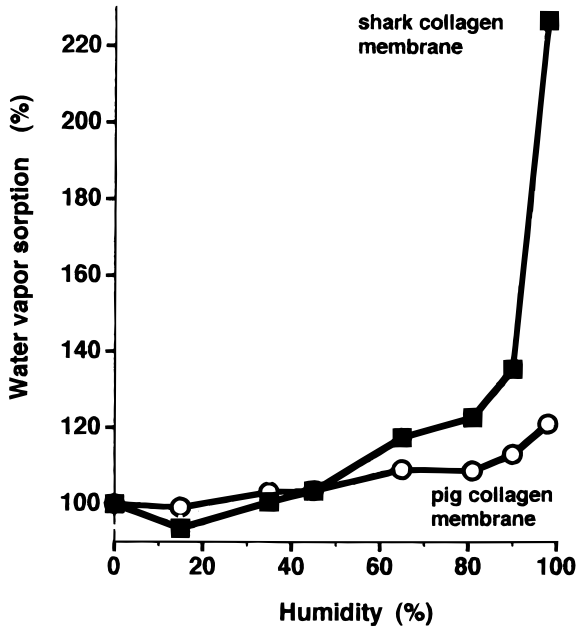

Figure 6. Water vapor sorption curves of collagen membrane.

extension than pig collagen membrane. The demonstrated sharp slope of the curve for shark collagen membrane means a greater gel strength, which suggests a well-grown network in shark collagen gel. This is consistent with the result of DLS that demonstrates a larger *D* value for shark collagen gel than from pig collagen gel, because an increased *D* is regarded as an indication of a decreased mobility of clustered collagen fibrils as discussed earlier.

Thus the difference in internal collagen gel structure of shark and pig collagen is supposed to result in the difference in mechanical strength of gel and membrane. As seen from the results of DLS and SEM, shark collagen gel is composed of more homogeneous fibrils and a network having more points of fibril-to-fibril interaction than pig collagen gel. Because the physical property of gel is generally parallel to that of the dried membrane from it, the harder shark collagen gel may contribute to the formation of a more stiffened membrane of shark collagen. Furthermore, the breaking strength of shark collagen gel increases much more rapidly with increasing collagen concentration than that of pig collagen gel. If an increase in collagen molecular concentration for shark collagen makes a contribution exclusively to the growth of collagen fibril diameter and hence does not make so great a contribution to the increase in the number of fibrils or fibril length, then the number of clusters cannot increase so much and hence cannot increase the breaking strength of gel. The data demonstrate a quite different state, that is, the increase in shark collagen concentration brings a rapid increase in the breaking strength of gel and produces rather thin fibrils as seen in SEM.

Water Vapor Sorption of Collagen Membrane. Water vapor sorption curves of collagen membrane were shown in Figure 6. The most striking feature is a sudden rise in water vapor sorption at 98% RH for shark collagen membrane. This indicates that water vapor can permeate more easily into shark collagen membrane and can expand the internal structure of membrane more strongly than pig collagen membrane at a high-moisture condition. This may be consistent with a stronger swelling capability of intact fibrils in shark skin and a stronger activity of electrolytic groups in fibrils to bind hydrogen ion and hydroxyl ion as reported previously by Yoshimura et al. (1996). The described strong water vapor sorption of shark collagen membrane probably reflects the feature in the internal structure of membrane that presumably is composed of thinner fibrils and hence contains a greater number of fibrils per volume or a longer length of fibrils as suggested by SEM and DLS studies, because such features must favor the membrane with a greater internal surface available to bind water vapor. The described strong water vapor sorption of shark collagen membrane reflects the thinner fibrils and the higher concentration of lattice points per unit by SEM and DLS, respectively. Namely, the surface areas of shark collagen fibrils were larger than those of pig collagen fibrils, and the water vapor sorption of shark collagen membrane was higher.

Conclusion. A summary of the physicochemical properties of shark collagen is as follows. (1) Denaturation temperature of shark collagen gel is about 41 °C (by DSC). (2) The diameter of fibrils in gel is relatively thinner and more homogeneous (by SEM). (3) Gel has a greater number of lattice points (by DLS). (4) Gel or membrane has a greater mechanical strength (by creep meter). (5) Collagen membrane has a higher water vapor sorption.

Taking in account that the in vitro fibrilogenesis of shark collagen proceeds in manner somewhat different from that of pig collagen (Nomura et al., 1997), the described features in physicochemical of shark collagen gel or membrane are probably related to the peculiar behavior of fibril formation for shark collagen. Consequently, shark skin collagen provides an unique material for uses in a form of gel or membrane in the field including food (table jelly, edible coating, etc.), cell culture and several uses as biomaterial.

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