Preparation and Dynamic Viscoelasticity Characterization of Alkali-Solubilized Collagen from Shark Skin

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Alkali-solubilized collagens, prepared by alkali−acid extraction and alkali direct extraction (abbreviated AASC and ALSC, respectively), were characterized by dynamic viscoelastic measurement of collagen solution (10 mg/mL). The optimum preparative conditions in terms of yield and polypeptide size are as follows: for the alkali−acid extraction, a pretreatment with 0.5 or 1 M NaOH containing 15% Na_2SO_4 within 5 days at 20 °C followed by the subsequent acid extraction, and for the alkaline direct extraction, a treatment with 0.5 M NaOH containing 10% NaCl at 4 °C for 20−30 days. A major portion of the polypeptide sizes of AASC and ALSC is composed of α chains (α1 and α2). Dynamic viscoelasticity of collagen solution was measured as a function of temperature. AASC showed a greater contribution of elastic behavior rather than viscous behavior. On the contrary, ALSC exhibits a stronger viscous behavior than elastic behavior.

Keywords: *Collagen; shark; rheology; dynamic viscoelasticity*

INTRODUCTION

Type I collagen, which is the major protein of skin and bone, is used in wide fields including foods, medicines, cosmetics, and cell cultures. Collagens from land animal origin are used mainly for the described uses; however, the development of collagen industries may demand the new collagen type I over the existing collagen. Shark skin has potential importance as a collagen resource. Shark is collected in connection with the tuna fishery and processed for foods in the fishery port; hence, a lot of fresh skin is generated as a byproduct. Type I collagen of shark skin is partially different in the molecular structure from those of land animals. Therefore, physicochemical properties of shark skin collagen should be investigated for its industrial use.

In a previous paper, we have demonstrated that the features of shark skin collagen, including solubility, denaturation temperature, swelling of fiber, reactivity to chemical reagents, and pepsin sensitivity, are different from those of bovine and pig (Nomura et al., 1995, 1997; Yoshimura et al., 1996, 1997). In particular, it is expected that the remarkable swelling potency of shark skin collagen plays an important role for the preparation of alkali-solubilized collagen. Since alkali attacks predominantly the telopeptide region of the collagen molecule, a highly alkaline solution has been used as a medium for collagen solubilization. This method has been developed on bovine hide, and some reports have been published (Crosby and Stainsby, 1962; Crosby et al., 1962; Hey and Stainsby, 1965; Fujii, 1969; Nakazaki et al., 1994). The most representative among them is the method using the following procedures. Bovine hide is soaked in 2−5% NaOH solution containing 15% Na_2SO_4 and amines at 4−20 °C for several days and subsequently extracted with dilute acid (Fujii, 1969). The main advantage of this method is that the purified collagen preparation shows good solubility at neutral solvent. However, by the alkaline treatment, functional groups in the helix region of the collagen molecule receive chemical modification partially. Furthermore, alkali-solubilized collagen shows a lower isoelectric point and denaturation temperature (Nakazaki et al., 1994). Since shark skin collagen is more sensitive to chemical reagents, the milder condition may be desirable for preparation of alkali-solubilized shark skin collagen. Therefore the present study was focused on the efficiency of alkali treatment and the physicochemical properties of the solubilized product. In addition, the dynamic viscoelasticity of collagen solution (10 mg/mL concentration) was measured in order to evaluate the material performance of shark skin collagen.

MATERIALS AND METHODS

Preparation of Collagen Cubes. Freeze-dried collagen cubes (about 5 × 5 × 5 mm) prepared from fresh great blue shark (*Prionace glauca*) skin corium according to a previous paper (Yoshimura et al., 1996) were used as material.

Solubilization by Alkali−Acid Extraction. This is a standard method for preparation of alkali-solubilized collagen. Collagen cubes about 100 mg were placed in 50 mL of NaOH solution (0.5 and 1 M) containing 5−15% Na_2SO_4 and shaken gently at 20 °C for 1−5 days. The cubes were then neutralized and washed with water. The cubes were extracted with 0.5 M acetic acid and the soluble fraction was dialyzed against 0.01 M acetic acid and then lyophilized. The product is abbreviated as AASC.

Solubilization by Alkali Direct Extraction. Collagen cubes (100 mg) were shaken in 50 mL of various concentration (0.1, 0.5, and 1 M) of NaOH at 4, 10, 15, and 20 °C, containing different types and concentrations of NaCl. A small portion of

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10.1021/jf990389d CCC: $19.00 © 2000 American Chemical Society
Published on Web 02/29/2000

the solution was taken at a specified interval and the protein content of supernatant was measured after centrifugation at 18 000 rpm for 20 min to evaluate the solubilization of collagen. Peptide chain composition of the dissolved collagen was determined by SDS−PAGE. From the collagen solution solubilized with 0.5 M NaOH containing 10% NaCl at 4 °C for 30 days, collagen was recovered by salting out, purified by redissolving in acid, dialyzed against 0.01 M acetic acid, and lyophilized. The resultant alkali-solubilized shark collagen (abbreviated as ALSC) was used in the experiment.

Analytical Methods. Protein content was determined by the microbiuret method. SDS−PAGE was carried out by the method of Laemmli (1970) for 10% gel and the method of Hayashi and Nagai (1979) for 5% gel.

Specific rotation was measured by the method of Nomura et al. (1995). Collagen was dissolved in 0.01 M acetic acid to give a concentration of about 2 mg/mL. After ultracentrifugation (Himac CP56GII, Hitachi, Japan) at 35 000 rpm for 60 min, the specific rotation of the sample was measured using a polarimeter (DIP-1000, JASCO, Japan) at 589 nm as the temperature was raised from 10 °C to 40 °C in 4 h. Dynamic viscoelasticity was measured using rheometer (ARES viscoelasticity measurement system, Rheometric Scientific). Collagen was dissolved in 0.1 M acetic acid to give a concentration of 10 mg/mL and was measured under the following conditions: frequency, 1 Hz; strain, 5%; heating rate, 0.5 °C/min; temperature range, 10−45 °C; geometry, 25 mm parallel plate. Amino acid composition of samples was determined on an automatic amino acid analyzer (JLC-300, JEOL, Japan) after hydrolysis in 6 M HCl at 110 °C for 24 h in sealed evacuated tubes.

RESULTS AND DISCUSSION

Solubilization by Alkali−Acid Extraction. Alkali-solubilized collagen is usually prepared by a combination of alkaline pretreatment and acid extraction as for cattle hide. The reason is that an alkaline treatment alone cannot dissolve a substantial part of hide collagen unless an extremely strong alkaline solution is adopted to lead the fragmentation of collagen chain. It is expected that for chemically sensitive shark skin collagen, a moderate condition is required. Alkaline pretreatment of shark skin collagen was carried out with 0.5 and 1 M NaOH solution containing Na_2SO_4 (5%, 10%, and 15%) at 20 °C, which is according to the method of Fujii (1969) with some modification. Time course of collagen dissolution in the pretreatment with changing the concentration of NaOH and Na_2SO_4 was followed (Figure 1). The solubility increased with time except for the case of 0.5 M NaOH/15% Na_2SO_4. The dissolution was rapid when the concentration of Na_2SO_4 was low. Any collagen dissolution in the pretreatment medium means a corresponding loss in collagen yield. Therefore, it is considered that all conditions involving the use of 0.5 M NaOH/0−10% Na_2SO_4 or 1 M NaOH/0−15% Na_2SO_4 except 0.5 M NaOH/15% Na_2SO_4 are unfavorable for alkaline pretreatment. Only a shortened period of alkaline pretreatment within 5 days with 1 M NaOH/15% Na_2SO_4 may be adopted.

The total dissolved protein by the alkaline pretreatment and acid extraction and the protein recovery from acetic acid extraction showed the highest value when the alkaline pretreatment was for 3 days (Table 1). The acid-extracted protein decreased when the alkaline pretreatment period was shorter. On the contrary, the protein loss in the pretreatment increased when the alkaline pretreatment period was longer. Consequently, pretreatment for 3 days at 20 °C is the optimum for alkali−acid extraction of shark skin collagen. All of the collagen preparations recovered from the acid extraction

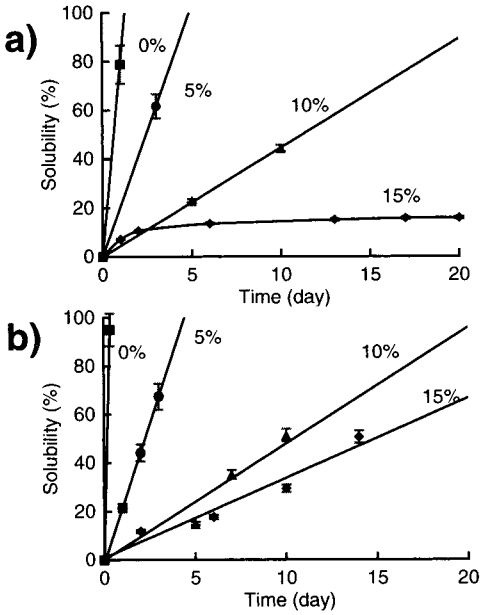

Figure 1. Solubilization of shark skin collagen treated with (a) 0.5 M and (b) 1 M NaOH containing varying concentration (0−15%) of Na_2SO_4 at 20 °C. Bars represent standard deviation of triplicate measurements.

Table 1. Solubilization and Recovery of Collagen with Alkali−Acid Extraction

pretreatment[a]		solubility (%)			
concn (M)	day	alkali[b]	acid[c]	alkali + acid[d]	recovery[e] (%)
1	1	8.8 ± 0.3	83.6 ± 4.1	92.4 ± 4.3	81.0 ± 2.6
	3	14.8 ± 0.4	84.7 ± 3.2	99.5 ± 3.7	79.2 ± 3.6
	5	15.5 ± 0.6	77.9 ± 3.6	93.4 ± 4.1	74.4 ± 3.1
0.5	1	5.8 ± 0.2	59.4 ± 2.4	65.2 ± 2.5	58.2 ± 2.1
	3	10.2 ± 0.3	87.6 ± 3.8	97.8 ± 4.2	82.9 ± 3.2
	5	11.0 ± 0.7	78.1 ± 3.1	89.1 ± 3.6	77.4 ± 2.9

[a] Alkaline pretreatment with 0.5 and 1 M NaOH containing 15% Na_2SO_4. [b] Solubility with alkaline pretreatment. [c] Solubility with acid extraction after alkaline pretreatment. [d] Total solubility of alkaline pretreatment and acid extraction. [e] Freeze-dried basis. Results represent the average and standard deviation of three different experiments.

of shark skin demonstrate α1 and β12 bands as main components and α2 and >β bands as minor bands on SDS−PAGE (Figure 2). The proportion of α chains increased as the alkaline pretreatment was prolonged. From the viewpoint of collagen yield (recovery protein in Table 1), taking into consideration that only a yield of about 15−30% has been reported for cattle hide with 2% or 3% NaOH/15% Na_2SO_4 pretreatment followed by acid extraction (Fujii, 1969), the present data with shark skin is a significant high yield. From the above results, it is concluded that a high yield of collagen from shark skin by alkali−acid extraction is attained by moderating the alkaline pretreatment condition established for cattle hide.

Solubilization by Alkali Direct Extraction. From the results of the earlier section, it was expected that an undegraded collagen preparation could be obtained from shark skin by alkali direct extraction. The effect of NaOH concentration and processing temperature was examined with respect to collagen solubility and SDS−PAGE pattern of the solubilized product (Figures 3 and 4). Treatment with 0.1 M NaOH at 4 °C dissolved collagen slowly, and the solubility reached only 10% for 20 days. The alkali treatment at 20 °C dissolved about 80% for 10 days. However, SDS−PAGE patterns of the

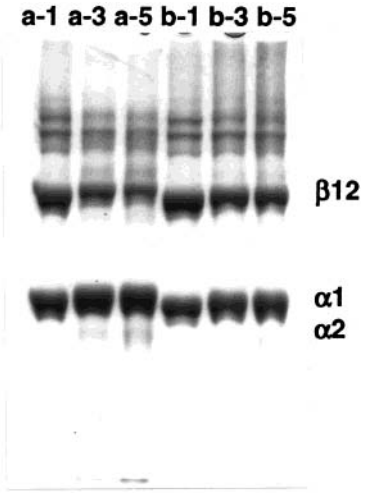

Figure 2. SDS−PAGE patterns of collagens prepared with alkali−acid extraction: (a-1, a-3, a-5) pretreatment with 1 M NaOH containing 15% Na_2SO_4 for 1, 3, or 5 days; (b-1, b-3, b-5) pretreatment with 0.5 M NaOH containing 15% Na_2SO_4 for 1, 3, or 5 days.

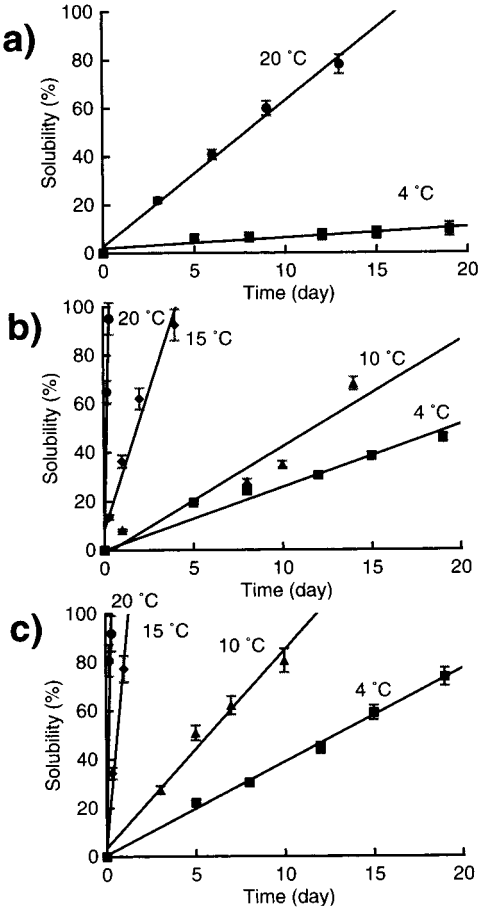

Figure 3. Solubilization of shark skin collagen treated with varying concentration (0.1−1 M) of NaOH containing 10% NaCl at varying temperature (4, 10, 15, 20 °C): (a) 0.1 M NaOH; (b) 0.5 M NaOH; (c) 1 M NaOH. Bars represent standard deviation of triplicate measurements.

solubilized product at 20 °C demonstrated no band of native collagen chains due to extensive cleavage of the peptide bonds. Treatment with 0.5 M NaOH at 4 °C dissolved collagen by about 50% for 20 days. SDS−PAGE of the solubilized product showed a major content of α and larger chains with only a limited amount of

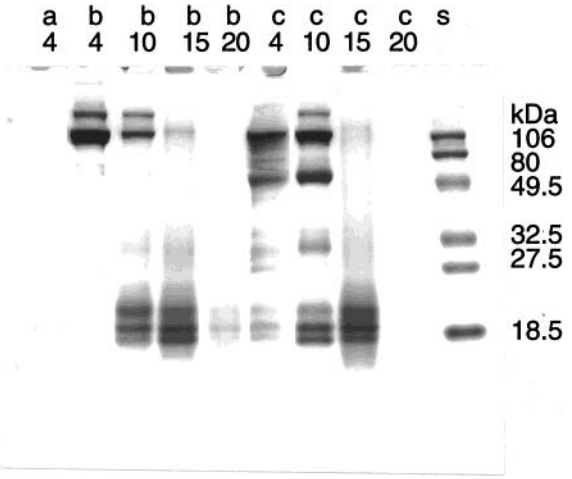

Figure 4. SDS−PAGE patterns of soluble fraction of shark skin collagen treated with varying concentration of NaOH containing 10% NaCl: (a-4) 0.1 M NaOH at 4 °C; (b-4) 0.5 M NaOH at 4 °C; (b-10) 0.5 M NaOH at 10 °C; (b-15) 0.5 M NaOH at 15 °C; (b-20) 0.5 M NaOH at 20 °C; (c-4) 1 M NaOH at 4 °C; (c-10) 1 M NaOH at 10 °C; (c-15) 1 M NaOH at 15 °C; (c-20) 1 M NaOH at 20 °C; (s) molecular mass of standard protein (kDa).

Table 2. Amino Acid Composition of Alkali-Soluble Fraction of Shark Skin Colllagen Treated with NaOH (0.5 and 1 M) Containing 10% NaCl

amino acid[a]	0.5 M		1 M			ASSC	PSSC
	1 day	4 days	1 day	4 days	7 days		
Hyp	19	23	36	44	49	67	69
Asp	103	100	90	76	67	41	41
Thr	52	50	45	37	30	22	23
Ser	60	59	57	50	46	43	42
Glu	106	107	99	92	92	75	74
Pro	67	69	75	80	87	111	111
Gly	128	146	177	216	247	321	323
Ala	70	75	81	96	104	123	124
1/2Cys	8	6	5	4	3	0	0
Val	48	48	40	39	38	23	24
Met	19	19	18	18	17	16	16
Ile	44	43	38	35	30	19	19
Leu	84	76	70	56	48	25	24
Tyr	38	34	29	24	22	3	1
Phe	36	35	31	28	26	14	13
His	22	22	19	16	14	9	9
Hyl	4	4	5	7	8	9	9
Orn	0	0	0	0	1	0	0
Lys	42	37	36	33	26	25	24
Arg	50	47	49	49	45	54	54

[a] Residues per 1000 amino acid residues. Results represent the average of three different experiments.

smaller fragments. Although treatment with 1 M NaOH at 4 °C dissolved collagen more quickly, a remarkable fragmentation of collagen was observed on SDS−PAGE pattern.

From the described results, treatment with 0.5 M NaOH containing 10% NaCl at 4 °C for 20−30 days seems to be the optimum for alkaline direct extraction from shark skin. However, the collagen solubility was about 50% under this condition and substantially lower than that of AASC. A condition to increase the solubility accelerates the fragmentation of collagen. Also the collagen purity of the solubilized product should be taken in consideration. Amino acid composition of the solubilized product demonstrates that noncollagenous protein (high content of Tyr) is released at the early stage of alkali extraction (Table 2). Therefore, it is desirable to separate the fraction extracted (about 10%

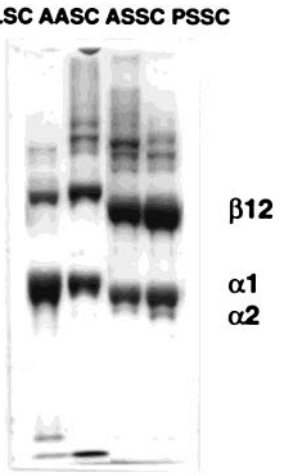

Figure 5. SDS−PAGE patterns of collagens.

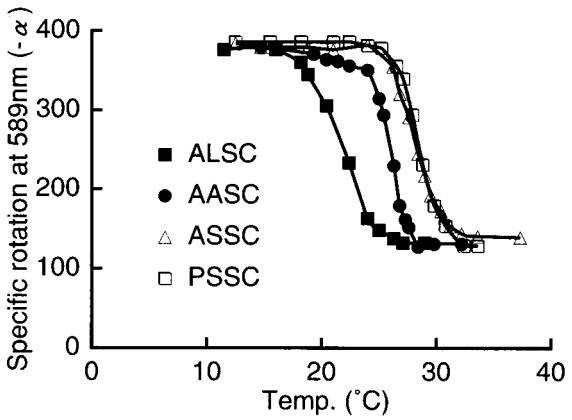

Figure 6. Denaturation curves of collagen preparations (ALSC, AASC, ASSC, and PSSC) measured by specific rotation.

estimated from Figure 1) in the initial 5 days of alkali treatment to be discarded, and then the subsequently extracted fraction is subjected to the preparation of ALSC.

Comparison of General Properties of Collagens. Chain composition of AASC and ALSC from shark skin was examined by SDS−PAGE (Figure 5). ALSC showed a remarkably high content of α chains and lower content of β chains compared with pepsin-solubilized shark skin collagen (PSSC) or acid-soluble shark skin collagen (ASSC). The α chain band in SDS−PAGE of ALSC probably contains a majority of the α1 chain and only a trace of the α2 chain because most of the α2 chain is combined with the α1 chain (to form the β12 chain) as reported in a previous paper (Kimura et al., 1981; Nomura et al., 1995). The chain composition of AASC positions at the intermediate of ALSC and PSSC or ASSC. This indicates that ALSC has received extensive cleavage of telopeptide and hence removal of interchain cross-link without the accompanying generation of low-molecular fragments. The level of the specific rotation of ALSC at 10 °C is almost equal to that of PSSC or ASSC (Figure 6) and suggests that the collagen helical conformation is conserved in ALSC.

The amino acid composition of collagen preparations demonstrated that ALSC and AASC showed a typical amino acid composition of type I collagen, like PSSC and ASSC, and that Tyr content decreased (data not shown).

The solubility of collagen preparations, ALSC, AASC, ASSC, and PSSC in a neutral solvent (50 mM phosphate buffer at pH 7.0), is shown in Table 3. The highest solubility (76%) of ALSC is remarkable. This feature of ALSC as well as its less tendency to form fibrils by self-assembly under neutral condition may be important in collagen use.

Table 3. Solubilization of Collagens in Neutral Solvents

collagen	solubilization[a] (%)	collagen	solubilization[a] (%)
ALSC	76.0 ± 2.1	ASSC	17.8 ± 0.6
AASC	16.0 ± 0.4	PSSC	51.0 ± 1.4

[a] Solubilization in 50 mM phosphate buffer at pH 7.0. Results represent the average and standard deviation of three different experiments.

The specific rotation−temperature curve of collagens is shown in Figure 6. Denaturation temperature of collagen is defined as the point at which specific rotation falls suddenly. Denaturation temperature of ALSC and ASSC was significantly lower than that of ASSC and PSSC and decreased in the order of ASSC, PSSC > AASC > ALSC. It seems that the lowest denaturation temperature of ALSC relates to a reduced interchain cross-link as shown in SDS−PAGE.

Dynamic Viscoelasticity Characterization of Alkali-Solubilized Collagen. Dynamic viscoelasticity technique gives information on the dynamic property of materials in processing through time analysis of the strain/stress response of polymer materials. In the present study, we applied this technique to collagen solution at a relatively high concentration (10 mg/mL). Storage modulus (G', elastic term), loss modulus (G'', viscous term), and tan δ (G''/G') of collagen solution were measured as a function of temperature (Figure 7). It has been reported that this technique is effective for characterizing rheologically pepsin-solubilized collagen (Yoshimura et al., 1999). G' and G'' of collagen solution suddenly fell and tan δ rose rapidly at a specific temperature for each collagen (AASC, ASSC, and PSSC). These temperatures for ASSC, AASC, and PSSC correspond to those of denaturation (helix−coil transition) temperatures by the specific optical rotation method (Figure 6). The decrease of G' and G'' and the increase of tan δ therefore show the collapse of the collagen triple helix to a random coil. On the contrary, ALSC showed a gentle viscoelastic change compared to other collagens for 10 mg/mL solution. However the viscoelastic property of 20 mg/mL ALSC solution showed a more clear change in G' and G'' similar to those of ASSC, AASC, and PSSC (Figure 8). The viscoelastic curve reflects clearly the denaturation (helix−coil transition) process for the four species of collagens. On the other hand, G' and G'' of AASC showed a high level, same as that of ASSC, and tan δ showed a low level in the undenatured state. This means that the viscoelastic property of AASC is similar to that of ASSC and their elastic behavior is substantially stronger than their viscous behavior. Unlike them, G' and G'' of ALSC showed a significantly low level and tan δ of ALSC showed a high level compared with those of AASC and ASSC in the undenatured state. This means that the viscoelastic property of ALSC is basically similar to that of PSSC and exhibits a greater contribution from the viscous behavior rather than the elastic behavior. In fact, tan δ of ALSC is somewhat higher than that of PSSC indicating a greater viscous contribution than PSSC. The most remarkable difference in rheological behavior of ALSC distinguished from other collagens is that the tan δ−temperature function of ALSC indicates a slow upturn of the tan δ

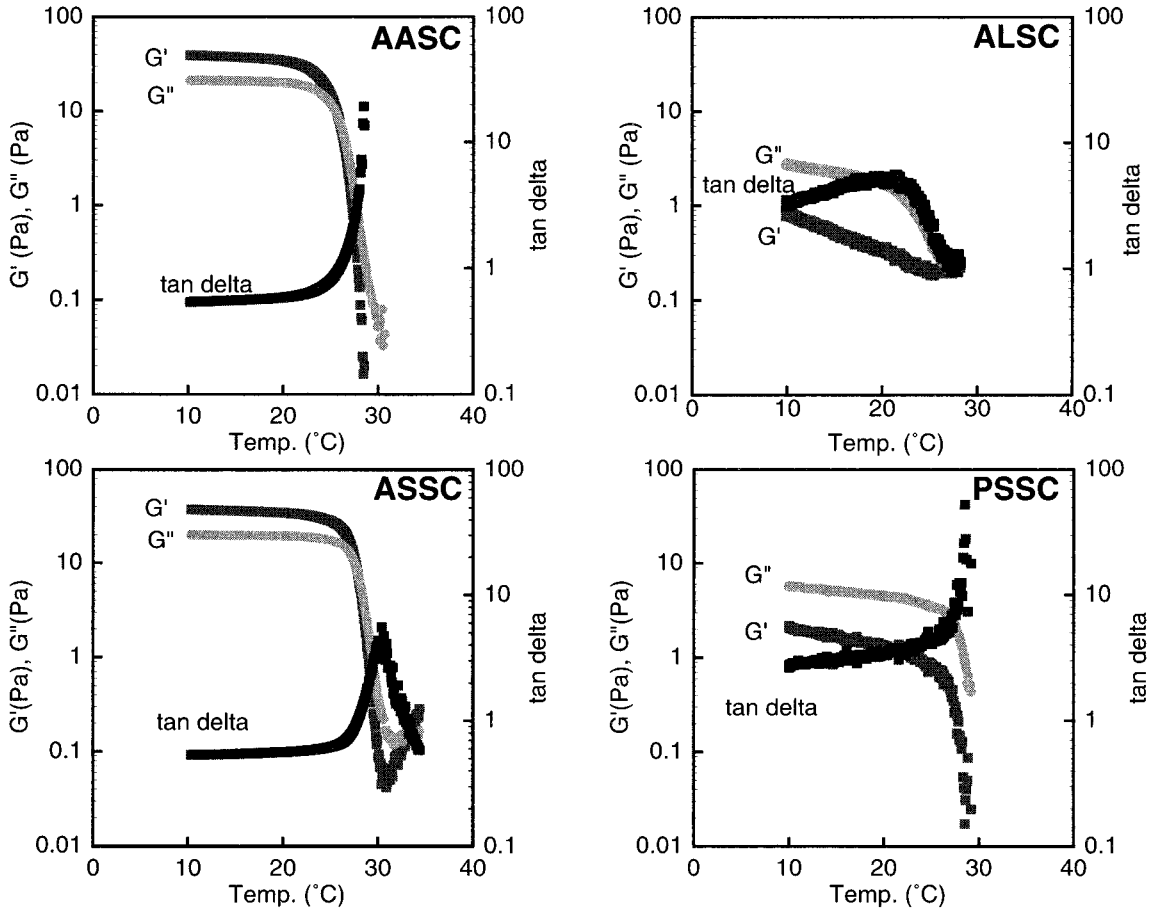

Figure 7. Dynamic viscoelasticity of collagens (10 mg/mL concentration of ALSC, AASC, ASSC, and PSSC) as measured by rheometer. The storage modulus G', loss modulus G'', and tan δ are plotted against temperature.

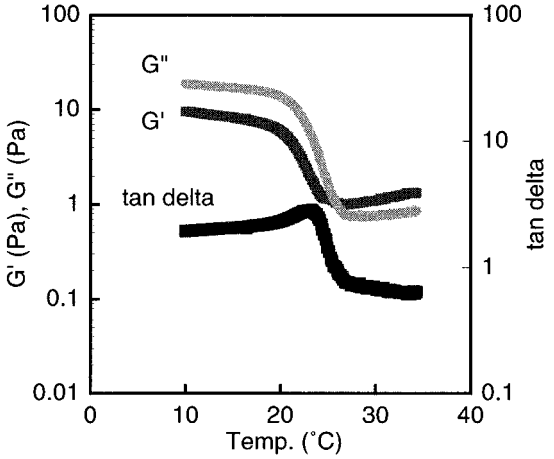

Figure 8. Dynamic viscoelasticity of collagen fluid gel of ALSC (20 mg/mL concentration) as measured by rheometer. The storage modulus G', loss modulus G'', and tan δ are plotted against temperature.

value rather than a rapid rise. Tan δ for 20 mg/mL ALSC also indicated a slow up turn of tan δ (Figure 8). This is somewhat surprising since the optical rotation study on ALSC demonstrates a typical rapid fall at denaturation temperature.

Another remarkable feature of ALSC is that G' decreases continuously with rising temperature indicating no clear turning point (Figure 7). This declining G' may reflect a decreasing tendency of intermolecular interaction with rising temperature and hence a significant temperature sensitivity of the physical property of ALSC due to its weak intermolecular interaction. All of the described results indicate the remarkable viscous character of ALSC solution. This prominent viscous character of ALSC probably provides an unique technical value of ALSC as collagen material.

We conclude that alkali-solubilized shark skin collagen, in particular ALSC, provides a new collagen material with a prominent viscous character for industrial use including foods and cosmetics.

ABBREVIATIONS USED

AASC, alkali−acid extracted shark collagen; ALSC, alkali direct extracted shark collagen; PSSC, pepsin-solubilized shark collagen; ASSC, acid-soluble shark collagen.

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Received for review April 21, 1999. Revised manuscript received September 13, 1999. Accepted December 2, 1999.

JF990389D