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Biochemical Properties of Black Drum and Sheepshead Seabream Skin Collagen

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Acid-soluble collagen (ASC) and pepsin solubilized collagen (PSC) were isolated from the skins of black drum (*Pogonias cromis*) and sheepshead seabream (*Archosargus probatocephalus*) harvested in the Gulf of Mexico coastal waters. The yields of ASCs on dry basis from black drum and sheepshead were estimated at 2.3 and 2.6%, and the yields of PSCs were 15.8 and 29.3%, respectively. Analyses of molecular weight profile, amino acid composition, and secondary structure showed that the skin collagens from both species were typical type-I collagen. The molecular mass of α_1 and α_2 subunits, as determined by SDS−PAGE using Tris-Acetate gels, was 127 kDa and 116 kDa, respectively. The amino acid composition of ASC and PSC for both species was closer to calf skin ASC than to cod skin ASC. Thermal denaturation temperatures, measured by melting point using circular dichroism, gave the following values: black drum ASC, 34.2 °C; sheepshead ASC, 34.0 °C; black drum PSC, 35.8 °C; sheepshead PSC, 34.3 °C. The literature value for the heat stability of calf skin collagen is 36.3 °C. The potentials of collagens from black drum and sheepshead skins in the functional food, healthcare, and pharmaceutical industries are discussed.

KEYWORDS: Collagen; fish; black drum; sheepshead seabream; denaturation; seafood wastes; skin; thermal stability

INTRODUCTION

The USDA has estimated that by the year 2025 more food and products for human health will be originating from the sea (*1*). At the present time, the seafood industry and regulators are eager to obtain full utilization of harvested aquatic-based food resources. Scientists, government agencies, conservationists, environmentalists, and seafood processors are calling for more efficient utilization of seafood and marine-based food products (*2*). Domestic landings of fish and shellfish in the Gulf States and adjacent waters were 0.8 million metric tons in 2000, and account for about 20% of the total landings in the U. S. (*3*). The state of Louisiana is "blessed" with abundant coastal and marine resources, and was ranked second only to Alaska in total landings (*3*). Louisiana seafood processors generate thousands of tons of aquatic-based food waste annually. As seafood processors in Louisiana strive to comply with environmental laws and learn about the potentials of obtaining health enhancing products from aquatic sources, interest is growing in obtaining higher value from their processing waste. This potential value for functional foods, fine biochemicals, and pharmaceuticals includes antimicrobials, antioxidants, antiinflammatory agents, enzymes, proteins, nucleic acids, calcium, oil, enzyme inhibitors, colors, pigments, dyes, and caviar, from fish, shellfish, and algal underutilized components.

Collagen is present in nearly all organs of vertebrates and is the major structural element of skin, bone, tendon, cartilage, blood vessels, and teeth. It is the most abundant protein in vertebrates, constituting about a quarter of the total. Collagen structure is distinguished by the formation of a right-handed triple superhelical rod consisting of three almost identical polypeptide chains. In addition, triple helical tropocollagen has the ability to associate laterally and longitudinally to form microfibrils. Each polypeptide chain forms a left-handed helix and consists of repeating triplets, $(Gly\text{-}X\text{-}Y)_n$, where X and Y are, with a high possibility, proline (Pro) or hydroxyproline (Hyp).

Nondenatured collagen derived from land-based animals (e.g., bovine and porcine) has been broadly exploited in functional food, cosmetics, and pharmaceuticals. However, due to the emergence of bovine spongiform encephalopathy (BSE), the foot-and-mouth disease (FMD) crisis, and the ban on European Union meat, the use of collagen and collagen-derived products from land-based animal skin has been called into question (*4*).

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Like other vertebrates, the skin of fish contains a high amount of collagen (*5*) and is abundantly available as a byproduct of fish fillet and surimi processing. There are many reports on thermal stability of fish collagen (*6*−*9*). Fish species with reported collagen denaturation temperatures above 30 °C are very limited (e.g., skipjack and carp). A majority of fish collagens, to date, denature below 30 °C, indicating that fish collagen is generally less stable than mammalian counterparts. There are very few fish species that contain collagen with high denaturation temperatures.

Collagen molecules in solution denature close to the upper limit of physiological temperature or the maximum body temperature of the animal species from which the collagen is extracted (*10*). Therefore, the tropical and subtropical fish inhabiting the Gulf of Mexico may contain collagen with denaturation temperatures above 30 °C.

Black drum and sheepshead are both subtropical fish with annual commercial landing of about a thousand metric tons each in Louisiana. The objective of this research was to investigate the biochemical properties and thermal stability of black drum and sheepshead seabream skin collagens.

MATERIALS AND METHODS

Materials. Black drum (*Pogonias cromis*) and sheepshead seabream (*Archosargus probatocephalus*) were harvested in the Gulf of Mexico. The fish were skinned and filleted in a seafood processing plant in Kenner, LA. The yield of skin from fish was about 10% according to the processing plant. The skins were then frozen and stored until analysis. Pepsin, EC 3.4.23.1, was obtained from Fisher Scientific (Fair Lawn, NJ). Calf skin type-I acid-soluble collagen was purchased from ICN Biochemicals Inc. (Irvine, CA). NuPAGE 3−8% gradient Tris-Acetate gels and staining and destaining kit were products of Invitrogen (Carlsbad, CA). All other chemicals were reagent grade.

Moisture Content. Moisture content was determined using OHAUS moisture determination balance Model 6010 (Florham, NJ).

Preparation of Acid-Soluble Collagen (ASC). All procedures to prepare ASC were carried out at ambient temperature (22−23 °C), except for the centrifugation operations that were performed at 4 °C. The skins were de-scaled and cut into small pieces (3 × 3 cm) with scissors. The scaled skins were suspended in 10 volumes of 0.1 M NaOH, and the suspension was stirred overnight with a magnetic stirrer. After decanting, the skins were re-suspended in 20 volumes of 0.1 M NaOH solution with stirring for 24 h. The alkaline insoluble components were strained through a cheesecloth and rinsed with distilled water repeatedly until a neutral pH was obtained. The insoluble components were extracted with 10 volumes of 0.5 M acetic acid for 3 days. The resulting viscous solution was centrifuged at 10 000*g* for 20 min at 4 °C. The residue was re-extracted with 10 volumes of 0.5 M acetic acid for 3 days, and the extract was centrifuged again. The supernatants of the two extracts were combined and were salted-out by adding NaCl to give a final concentration of 0.9 mol dm^{-3}. After standing overnight, the resulting precipitate was collected by centrifuging at 10 000*g* for 20 min. The precipitate was dissolved in 10 volumes of 0.5 M acetic acid. Salting-out and solubilization procedures were repeated 3 times. The resultant solution was dialyzed against 0.1 M acetic acid for further experiments.

Preparation of Pepsin-Solubilized Collagen (PSC). All PSC preparation procedures were performed at ambient temperature (22−23 °C), except for the centrifugations, which were performed at 4 °C. After de-scaling and cutting the skins, the alkaline-treatment procedures were performed similar to the preparation of ASC. After neutralization, the insoluble components were solubilized with 10 volumes of 0.5 M acetic acid containing 0.1% (w/v) pepsin for 3 days. The resulting viscous solution was centrifuged at 10 000*g* for 20 min at 4 °C. The residue was re-extracted with 10 volumes of 0.5 M acetic acid solution containing 0.1% (w/v) pepsin for 3 days, and the extract was centrifuged again. The supernatants of the two extracts were combined and were salted-out by adding NaCl to give a final concentration of 0.9 mol dm^{-3}. After standing overnight, the resulting precipitate was collected by centrifugation at 10 000*g* for 20 min. The precipitate was dissolved in 10 volumes of 0.5 M acetic acid. Salting-out and solubilization procedures were repeated 3 times. The resultant solution was dialyzed against 0.1 M acetic acid.

Circular dichrorism (CD). The prepared collagen samples were diluted to a concentration of 0.3 g dm^{-3} with 0.1 M acetic acid solution. CD measurements were made with an AVIV circular dichroism spectrometer 62 DS (Lakewood, NJ) calibrated with re-crystallized d-10-camphorsulfonic acid, $[\theta]_{290.5} = 7800$ (deg cm^2 $dmol^{-1}$). The CD spectrum of the sample was measured using a 0.1-cm path-length quartz cell. Five scans were averaged for the wavelength 250−195 nm. A mean amino acid residue weight of 91 g mol^{-1} for collagen (*11*) was assumed to determine its molar ellipticity $[\theta]$. The melting curve of collagen was determined by monitoring $[\theta]$ at the wavelength of a positive extreme at 220 nm, which is characteristic of the CD spectrum for collagen triple helix. The $[\theta]_{220}$ was recorded while heating the sample at the rate of 0.5 °C/min from 15 to 45 °C. The transition temperature, T_m − CV, was determined as the midpoint temperature between native-folded and completely unfolded forms. The analyses were repeated twice. The repeatability of the temperature measurement was better than 0.1 °C.

Viscosity Measurements. Collagen samples (0.3 g/dm^3) were prepared in the same manner as the CD sample. A Cannon−Fenske type Kinematic Viscometer tube (Fisher Scientific) with the flow time for water of 120 s at 25 °C was employed for the viscosity measurement. Collagen solution (8 mL) was incubated for 30 min at a given temperature from 15 to 45 °C and then its viscosity was determined by measuring the flow time at the same temperature. The measurement was repeated three times. Specific viscosity (η_{sp}) was calculated by the equation $(t - t_0)/t_0$, assuming the densities of the solution and solvent were the same (t = flow time of the collagen solution and t_0 = flow time of the solvent). Thermal denaturation curve of the collagen solution was depicted by plotting the reduced viscosity (η_{sp}/c), where c is the protein concentration (g/dm^3), against temperature. Thermal denaturation temperature, T_d-V, was expressed as a midpoint temperature between the extrapolated line for native collagen and that for fully denatured collagen on the (η_{sp}/c) vs temperature plot.

Sodium Dodecyl Sulfate-Gel Electrophoresis (SDS-PAGE). Electrophoresis was carried out using NuPAGE Tris-Acetate gel (3−8%). Dialyzed samples were heated at 100 °C for 5 min in NuPAGE LDS sample buffer (Invitrogen) including NuPAGE reducing agent (Invitrogen). The electrophoretic separation was carried out according to the manufacturer's procedure. The gel was stained using NOVEX colloidal blue staining kit (Invitrogen) for 8 h with shaking. After staining, the staining solution was decanted and replaced with 400 mL deionized water overnight with shaking. Band intensities of the gel were analyzed using Scion Image Version Beta 4.0.2 (Scion Corp., Frederic, MD).

Protein Determination. The modified Biuret method of Umemoto (*12*) was employed. The nitrogen-protein conversion factor for collagen is 5.4 (*13*).

Amino Acid Composition. Samples were hydrolyzed under vacuum with 6 N HCl at 110 °C for 24 h under argon atmosphere in the presence of phenol. Amino acid analysis was performed using a Hewlett-Packard AminoQuant II system (Palo Alto, CA). The analyses were repeated three times, and the results were reported as the mean ± standard deviation.

RESULTS AND DISCUSSION

Chemical Properties. Acid-soluble and pepsin-solubilized collagens were isolated from black drum and sheepshead seabream skins. **Figure 1** shows SDS−PAGE patterns of the isolated collagens. The black drum ASC as well as the sheepshead ASC had at least two different α chains (α_1 and α_2) and their cross-linked chains (dimer is referred to as β, trimer is referred to as γ). In both species, the molecular mass of each subunit was 127 kDa for α_1 and 116 kDa for α_2, respectively. The band of α_1 might contain the α_3 component that has molecular mass indistinguishable to α_1 on an SDS-PAGE gel,

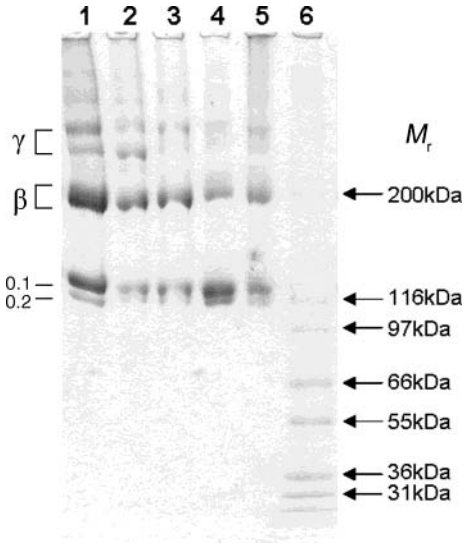

Figure 1. SDS-PAGE patterns of collagens. (1) Calf skin type-I acid soluble collagen (ASC), (2) black drum ASC, (3) sheepshead ASC, (4) black drum pepsin solubilized collagen (PSC), (5) sheepshead PSC, and (6) molecular weight markers.

Table 1. Band Intensity Ratio of Cross-Linked Chain to Total Monomer Chains in Collagen

	calf skin ASC	black drum ASC	sheepshead ASC	black drum PSC	sheepshead PSC
$\beta/(\alpha1+\alpha2)$	1.26	1.59	1.30	0.50	0.84
$\gamma/(\alpha1+\alpha2)$	0.75	1.32	0.62	0.23	0.51

resulting in the co-presence of $\alpha_1\alpha_2\alpha_3$ as a minor component together with $[\alpha_1]_2\alpha_2$ as a major component (*7*). These values were similar to the molecular mass of calf skin type-I acid-soluble collagen subunits. The existence of at least two different subunits confirmed that the major collagen from the fish skins is the type-I collagen (*14*). PSC α chains, and ASC α chains were similar in electrophoretic mobility, suggesting that cleavage sites by pepsin were located very close to the terminal ends of the tropocollagen. **Table 1** shows the band intensity ratio of cross-linked chain (β or γ) to total non-cross-linked monomer chains ($\alpha_1 + \alpha_2$) on the SDS-PAGE gel. Black drum ASC contained the highest population of cross-linked components of all collagens samples including calf skin type-I ASC. The intra- and/or intermolecular cross-linking of collagens, β and γ components, were richer in ASC than in PSC. This greater cross-linking was explained by conversions of some β- and γ-chains in fish skin collagen matrix to α-chains by the action of pepsin. Pepsin removes the cross-link-containing telopeptide, and the concomitantly one β-chain is converted to two α-chains (*15*). The yields of ASCs on dry basis from black drum and sheepshead skins were estimated at 2.3 and 2.6%, respectively, and the yields of PSCs were 15.8 and 29.3%, respectively. The high yields observed in PSCs are due to the improvement of collagen extractability by use of the acid protease, as reported by Nagai et al. (*16*).

The amino acid composition of the collagens is shown in **Table 2**. Black drum and sheepshead skin collagens had similar amino acid profile. The collagens were rich in proline (Pro), glycine (Gly), and alanine (Ala), which are characteristic of all collagens. High levels of hydroxylysine (Hyl) and hydroxyproline (Hyp), similar to collagens from mammalian species, were measured in black drum and sheepshead collagens. The distribution patterns of amino acid composition were closer, especially for Pro and Hyp, to calf ASC rather than to cod ASC (**Table 1**).

Table 2. Amino Acid Composition of Skin Collagens[a]

	black drum		sheepshead		cod	calf
	ASC	PSC	ASC	PSC	ASC[b]	ASC[c]
Hyp	80.2 ± 7.0	89.7 ± 6.2	87.2 ± 10.4	89.2 ± 3.7	40.7	94
Asx	43.9 ± 1.1	45.9 ± 3.9	43.9 ± 4.1	45.7 ± 5.1	41.5	45
Thr	25.9 ± 0.6	27.4 ± 3.6	26.8 ± 3.3	27.2 ± 1.6	26.1	18
Ser	40.7 ± 2.3	44.6 ± 10.5	42.5 ± 4.3	44.7 ± 3.0	60.8	33
Glx	70.1 ± 2.3	70.7 ± 9.5	68.4 ± 6.6	67.3 ± 3.2	77.4	75
Pro	119.6 ± 2.7	107.4 ± 21.3	117.9 ± 10.5	108.9 ± 4.3	89.6	121
Gly	319.8 ± 9.8	314.6 ± 7.4	320.6 ± 21.2	321.1 ± 16.2	332.2	330
Ala	128.7 ± 2.4	130.8 ± 18.8	131.9 ± 10.4	127.7 ± 7.1	105.6	119
Cys	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ND	ND
Val	19.6 ± 0.5	19.5 ± 1.8	18.8 ± 1.5	18.8 ± 0.8	25.2	21
Met	14.2 ± 0.5	14.4 ± 1.2	13.5 ± 0.8	15.3 ± 0.3	20.6	6
Ile	6.9 ± 0.4	7.6 ± 0.8	6.5 ± 0.9	7.7 ± 0.9	17.1	11
Leu	20.8 ± 0.5	21.2 ± 2.3	18.8 ± 2.2	19.6 ± 1.6	29.7	23
Tyr	4.0 ± 0.3	0.0 ± 0.0	2.9 ± 0.1	1.4 ± 2.4	5.0	3
Phe	13.8 ± 0.8	13.9 ± 0.9	13.4 ± 1.1	14.0 ± 0.5	13.8	13
Hyl	5.8 ± 0.9	10.3 ± 2.4	6.3 ± 1.1	8.4 ± 2.5	7.6	7
Lys	23.5 ± 2.8	22.9 ± 5.9	22.0 ± 4.3	23.4 ± 0.8	33.1	26
His	6.0 ± 0.5	3.7 ± 3.2	4.9 ± 0.3	5.6 ± 0.6	11.8	5
Arg	56.5 ± 1.1	55.5 ± 9.4	53.8 ± 3.3	54.0 ± 3.0	62.5	50
total	1000	1000	1000	1000	1000	1000

[a] The mean ± standard deviation of three determinations for the same sample preparations. ND, not determined; Asx = Asp + Asn; Glx = Gln + Glu. [b] Yamaguchi et al. (*22*). [c] Herbage et al. (*23*).

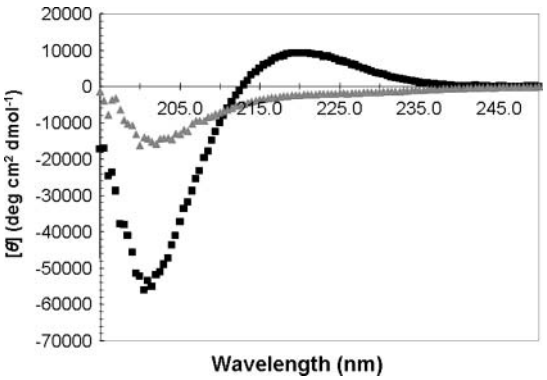

Figure 2. Circular dichroism spectra of black drum skin ASC at different temperature (square, 15 °C; triangle, 45 °C). The spectrum was recorded after 30 min incubation at the respective temperature.

Secondary Structure. Figure 2 shows a representative CD spectrum of collagen used in this study. The native black drum ASC sample, at 15 °C, gave a characteristic CD spectrum with a positive extreme at 220 nm and a negative peak at 201 nm. The CD spectrum of sheepshead ASC showed a similar profile to black drum ASC CD spectrum. CD spectra of ASC and PSC were very similar to that of calf skin collagen (*17*). These spectra are characteristic of a collagen triple-helix structure (*18*). On heating at 45 °C for 30 min, the peak around 220 nm flattened and the depression at 201 nm was lifted up. These spectral changes reflect the thermal transition of the triple-helix form to a random coiled form. The spectrum of the thermally denatured collagen was never restored to the nondenatured spectrum on cooling (data not shown), demonstrating that the denaturation was irreversible.

Thermal Stability Studies. The melting curve of collagen triple-helix is shown in **Figure 3**. Collagen helices melted with increasing temperature. The melting curves of PSC were an apparently bi-phase transition in contrast to an apparently mono-

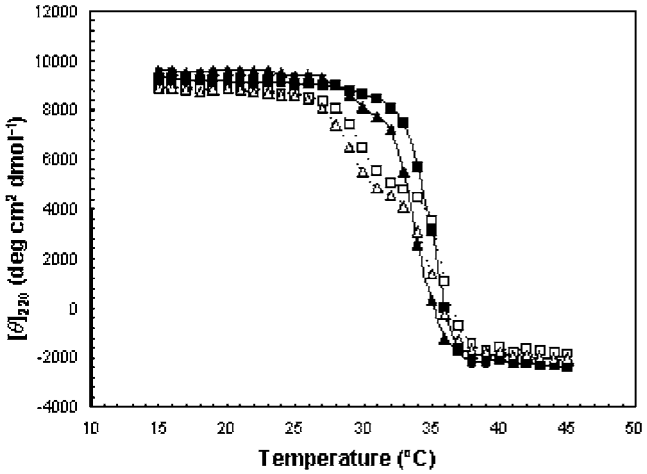

Figure 3. Melting curves of collagens. Filled square with solid line, black drum ASC; filled triangle with solid line, sheepshead ASC; unfilled square with dotted line, black drum PSC; unfilled triangle with dotted line, sheepshead PSC.

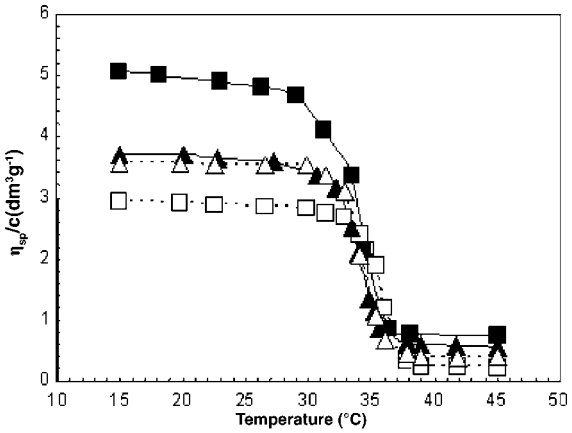

Figure 4. Thermal denaturation curves of collagens measured by viscosity. Filled square with solid line, black drum ASC; filled triangle with solid line, sheepshead ASC; unfilled square with dotted line, black drum PSC; unfilled triangle with dotted line, sheepshead PSC. The experimental data were shown in mean values and error bars represent standard deviations ($n = 3$).

phase transition for ASC. This suggests that the PSC collagen possesses at least two inner domains with diverse stabilities, or two different collagen molecules with diverse stabilities (e.g., the coexistence of $[\alpha_1(I)]_2\alpha_2(I)$ and $\alpha_1(I)\alpha_2(I)\alpha_3(I)$) or that of tropocollagen and atelocollagen. Such a two-stage melting occurred in calf skin (*15*), adult bovine derma, and rat-tail tendon collagens (*17*) as well. It was reported that the thermal transition pattern of collagen is affected by heating rate (*18*), as well as by protein concentration (*17*, *19*). The first-phase transition for PSC began at about 25 °C, while the transition for ASC started at 28 °C for black drum and 27 °C for sheepshead, respectively. The transition was completed near 38 °C for both black drum and sheepshead collagens. The melted collagen formed a random coil, as shown by the CD spectrum at 45 °C (**Figure 2**). Transition curves for black drum collagens appeared to shift at a temperature near 1.5 °C higher than those of sheepshead collagens.

One physicochemical characteristic of collagen is high viscosity. **Figure 4** shows the thermal behavior of collagen viscosity. Reduced viscosity at 15 °C was higher for ASC than that of PSC. Black drum ASC showed higher viscosity than

Table 3. Imino Acids Content and Thermal Transition Temperature of Collagens

source of collagen	imino acids (Pro + Hyp) content per 1000 residues	T_m-CD/ °C	T_d-V/ °C	physiological temp [a]/°C
black drum skin ASC	199.8	34.8	34.2	15–35
sheepshead skin ASC	205.1	33.5	34.0	10–35
black drum skin PSC	197.1	35.1	35.8	15–35
sheepshead skin PSC	198.1	33.6	34.3	10–35
cod (*Gadus morhua*) skin ASC	130.3	13.0[c]	ND[b]	–0.5–10
calf skin ASC	215	ND	36.3[d]	37[e]

[a] Marine and Coastal Species Information (*21*). [b] ND, not determined. [c] Burge and Hyness (*6*). [d] Sikorski et al (*5*). [e] Body temperature.

the other three collagen molecules. The greater viscosity can be accounted for by the high average molecular weight of black drum ASC, which results from the high proportion of β- and γ-chains (**Table 1**). The viscosity of the ASCs declined as temperature increased from about 27 to 39 °C, while the viscosity of the PSCs decreased from about 30 to 39 °C. Thermally lowered viscosity could not be recovered upon cooling (data not shown). The thermal pattern of decreasing viscosity was expected to be similar to that shown in the melting curves in response to heat-induced protein denaturation. However, small temperature effect differences were observed between the CD spectrometer and viscometer data, resulting from the differences in measurement conditions, such as heat treatment.

Correlation between Chemical and Stability Characteristics. Table 3 summarizes imino acids (Pro + Hyp) content and thermostability results with physiological temperature of fish. In general, collagen has a high content of imino acids, and imino acid content is closely related to thermostability (*10*). The content of imino acids of black drum and sheepshead collagen was closer to the imino acid content of calf collagen than of cod, which inhabits cold-temperature sub-arctic waters (**Table 3**). The melting temperature determined using CD was 34.8, 35.1, 33.5, and 33.6 °C for black drum ASC, black drum PSC, sheepshead ASC, and sheepshead PSC, respectively. These temperatures are similar to the denaturation temperature, T_d-V, range, 34.0−35.8 °C, of the Gulf coast fish studied. There was no significant difference in T_d-V between the collagens of the two fish species and ASC and PSC. The high denaturation temperatures (34.0−35.8 °C) indicated that black drum and sheepshead collagens have high thermal stability similar to calf skin collagen (36.3 °C) (*4*). Denaturation temperatures of collagens from over 30 marine sources (including 25 Teleostei species) have been determined under similar conditions (0.1 M acetic acid as solvent and protein concentration of 0.3 g dm^{-3}) (*7*−*9*). The denaturation temperatures of black drum and sheepshead collagens were higher than all of the fish collagens previously reported. Aquatic species collagen that denatures above 30 °C includes skipjack (33.0 °C), carp (32.5 °C), eel (30.2 °C), and Japanese sea bass (30.0 °C) (*7*−*9*). Thus, black drum and sheepshead skin collagen was found to be quite heat-stable.

It is well-known that the transition temperature of monodisperse collagen is close to the upper limit of physiological temperature (*10*, *20*). Subtropical fish species, like black drum and sheepshead, are capable of inhabiting water at temperatures above 30 °C (*21*). The high thermal stability of black drum and sheepshead collagen results from the high content of Pro and Hyp, allowing adaptation to high water temperatures. The correlation between protein stability and upper physiological

temperature suggests that other fish species living in the Gulf of Mexico coastal waters may also have thermally stable collagens. The high denaturation temperature of black drum and sheepshead collagens indicates the possible substitution of these marine collagens for the land based animal collagens in current commercial applications.

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