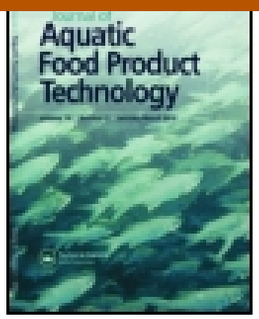

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Characterization of Collagen from Different Discarded Fish Species of the West Coast of the Iberian Peninsula

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ABSTRACT

Skin collagen of six discarded fish species was analyzed and compared. Acid soluble collagen (ASC) was extracted; a characteristic sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile for type I collagen was obtained, except for *Chimaera mostrosa*. Contents of collagen calculated from HPro (31.85% average) were higher than those determined from ASC extracts (17.75% average), with *Galeus* spp. being the species with the higher percentage. Amino acid analysis revealed the typical composition of collagen, with very few differences among species. Specific profiles were obtained after protease digestion. Denaturation temperature of ASC correlated well with imino and hydroxyproline contents.

Results demonstrate the feasibility of using the obtained collagens in different industrial applications.

KEYWORDS

Fish skin; discards; collagen; hydroxyproline

Introduction

Collagen, the main structural protein of metazoans, is present in several types of tissues such as skin, bone, cartilage, etc. (Shigeru and Masataka, 1999). Collagens comprise different molecular species or types; in the case of fish, collagen type I is a family of molecules mainly present in the skin (Bailey et al., 1998). These molecules present a quaternary structure involving three polypeptides (alpha chains), each twisted in left-handed helix and arranged together in right-handed super-helix structure; this is stabilized by the existence of interchain crosslinks (Bailey et al., 1998). Fish collagens type I are typically constituted by two alpha chains, α1 and α2, of similar molecular weight of about 100 kDa (Gómez-Guillén et al., 2002; Kimura and Ohno, 1987); however, the existence of an α3 subunit in some fish species has also been described (Saito et al., 2001; Kimura et al., 1987). The partial hydrolysis of native collagen produces gelatin, which is the usual form in which collagen is industrially prepared.

Collagen and gelatin have different industrial applications, among the best known are the food, cosmetic, biomedical, and film industries (Karim and Bhat, 2009). The main production of collagen relies on skin and bones from pig and cattle; however, since the outbreak of bovine spongiform encephalopathy (BSE), some concerns have arisen about collagen and gelatin from cattle, and more attention has been paid to its production from alternative sources.

Wastes from fish can represent an average of 55% of the total fish weight; from this material, up to 30% can be skin and bones. In a country like Spain, where landed fish, fresh and frozen, was about 654,000 t during 2009, fish wastes could represent 360,000 t and up to 107,910 t of bones and skins (Blanco et al., 2007).

These numbers show that an important amount of this unused biomass could be employed to produce some valuable compounds, such as gelatin or collagen. Furthermore, besides fish

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industrial subproducts, it is a well-known fact that an even higher amount of fish and other marine organisms are discarded at sea (Kelleher, 2005). Fisheries discards represent an important problem. Therefore, the European Commission is discussing a reform of the European common fisheries policy, which includes the prohibition of discard practices (http://ec.europa.eu/fisheries/reform/docs/discards_en.pdf). One important aspect of the policy is to find alternatives for dealing with discards; this may include the valorization of the whole fish or components of them. Fish collagen has been extracted mainly from fish skin; several works have addressed the use of fish skins as sources of collagen and gelatin (Senaratne et al., 2006; Nagai and Suzuki, 2002; Zhang et al., 2009; Gómez-Guillén et al., 2002). To our knowledge, this is one of the first studies reporting the extraction of skin collagen from discarded or underutilized fishes. We assumed that the possible differences found in the collagen extracted are consequences of fish species. The present work deals with the characterization of the collagen from the skin of five Chondrichthyes (rabbit fish: *Chimaera monstrosa*, lantern shark: *Etmopterus* spp., catshark: *Galeus* spp., small-spotted catshark: *Scyliorhinus canicula*, and cuckoo ray: *Leucoraja naevus*) and one teleost (common Atlantic grenadier: *Nezumia aequalis*) never reported before. These species present a high discard rate, either because they do not have any commercial value or because the value is often quite low, making it not profitable to bring those fish to shore. We interviewed local fishermen, and the results indicated that the discard level for some of the species studied here can be up to 100% in nearby waters of northern Spain. Collagen extraction from the skin of cartilaginous fish can be expected to produce a significant collagen yield due to the fact that skin plays an important role in maintaining body form in these animals, which do not have a strong skeleton like bony fish. Therefore, skins are usually thicker compared with other noncartilaginous fish (Hwang et al., 2007). Some of the low value sharks, like small-spotted catshark (*S. canicula*), are consumed as human food; but skins are usually removed by fish mongers. This is a subproduct of these species that is normally wasted.

Among the Chondrichthyes—which includes sharks, skates, and rays—there are some studies reporting data on properties of type I collagen (Yoshimura et al., 2000; Nomura et al., 1997). As far as we know, this is the first study about skin collagen from these species, such as rabbit fish (*Chimera monstrosa*) or small-spotted catshark (*S. canicula*). Furthermore, it has been previously reported that shark skin collagen provides an unique material for different purposes as biomaterial, in the feed industry, or cell culture (Nomura et al., 2000). Most collagen properties—such as gelling ability, viscosity, or thermal denaturation—depend on their amino and imino acid composition. All these characteristics, together with the yield and denaturation degree, can be used to define the final use of the collagen or gelatin extracted from fish subproducts. The aim of this work was to extract acid soluble collagen from discarded and not valuable species for feed purpose and to characterize them to provide information about the suitability for a future use of this extracted collagen in several applications such as food, cosmetics, or biomedical.

Material and methods

Fish species

Discarded fish were obtained from a trawler vessel which operates in the West coast of the Iberian Peninsula (FAO IXa, in particular 40º 41′ N, 9º 17–45′ W). Fish arrived fresh and iced to our laboratory where they were introduced in plastic bags, frozen, and stored at −20ºC (for up to 6 months) until analysis. Several specimens (see Table 1) of the following fish species were used in this study: rabbit fish (*Chimaera monstrosa*; CMON), cuckoo ray (*Leucoraja naevus*; LNAE), common Atlantic grenadier (*Nezumia aequalis*; NAEQ), lantern shark (*Etmopterus* spp.; ETMO), catshark (*Galeus* spp.; GAL), and small-spotted catshark (*Scyliorhynus canicula*; SCAN; Figure 1). Skin from up to three specimens of each species was manually obtained; skins were used for collagen extraction and skin characterization.

Table 1. Composition of skin from discarded fish.

Species	% Water	% Protein (dry basis)	% Extracted collagen (dry basis)
Nezumia aequalis (2)	59.63 ± 0.58	41.38*	20.98 ± 4.63 a,c
Chimaera monstrosa (3)	73.01 ± 3.98	30.21 ± 5.23	20.09 ± 4.30 a,c
Etmopterus spp. (2)	69.07 ± 3.82	23.27 ± 2.17	5.81 ± 2.84 b
Galeus spp. (3)	61.85 ± 2.64	32.07 ± 2.74	14.04 ± 1.55 a,b
Scyliorhinus canicula (3)	62.89 ± 0.69	30.73 ± 2.44	16.48 ± 3.51 a,b
Leucoraja naevus (2)	71.37 ± 0.78	28.25 ± 0.77	29.10 ± 4.34 c

Numbers in parentheses represent the number of individuals used for the study. ANOVA analysis was performed for extracted collagen showing
significant differences among species ($F = 3.796$, $p < 0.05$). Same letter indicates no statistical difference among means ($p < 0.05$).
*Data for one specimen.

Identification of fish species was performed by morphological characterization and also by DNA analysis. In this case, DNA was extracted from a portion of the muscle of each species, and mitochondrial DNA analysis was performed (Blanco et al., 2008; Calo-Mata et al., 2003).

Analytical methods

Acid extraction of collagen

Acid extraction collagen was carried out according to the methodology of Hwang et al. (2007) with slight modifications. Skin of fish was cut into small pieces. About 10 g of this material was weighed and mixed with 10 volumes of 0.1 M NaOH and stirred in a cold room (3–5°C) for 24 h. Liquid was removed, and the residue was washed with distilled water (three times). Washed residue was stirred overnight with 10 volumes of 0.5 M acetic acid. After this, the extract was centrifuged at 10,000 × *g* for 20 min at 10°C. Supernatant was dialyzed against distilled water for 2 days in a cold room, at 3–5°C. Volume of dialyzed collagen extract was measured and freeze-dried. Freeze-dried collagen was referred to wet weight or freeze-dried skin.

Determination of collagen content

Hydroxyproline (HPro) was used as an estimation of collagen. Since this amino acid is highly represented in collagen and elastin, it is assumed that all HPro content of skin is due to collagen, and the ratio of HPro in collagen is 12.5 g of HPro/100 g of collagen (Edwards and O'Brien, 1980).

Thirty milligrams of dry skin were weighed and introduced in hydrolysis microwave tubes, and 4 mL of 6 M HCl were added. Hydrolysis was performed in a microwave (Berghof speed wave MWS-2 microwave; Berghof, Eningen, Germany) at 150°C for 90 min at 70% power. Once the hydrolysis step was complete, samples were allowed to cool down to room temperature and were made up to a known volume with 6 M HCl. Four-hundred microliters of this solution were transferred to glass vials and let dry in a vacuum desiccator at 60°C in presence of solid NaOH, with vacuum drawn for 24–48 h. The resulting dry matter was suspended in 8 ml of buffer (0.13 M citric acid, 0.75% glacial acetic acid, 0.6 M sodium acetate, 0.15 M sodium hydroxide, and 20.13% n-propanol; pH was adjusted to 6.5 with 0.2 M NaOH; and volume was brought to 660 mL with distilled water).

Hydroxyproline primary standard was prepared by dissolving 50 mg of hydroxyproline (Sigma, St. Louis, MO, USA) in 100 mL of buffer. From this primary standard, a calibration curve of hydroxyproline, ranging from 0.5 µg/mL up to 10 µg/mL, was prepared. Chloramine-T reagent is prepared just before its use (0.05 M Chloramine in distilled water). Three milliliters of either samples or standards were placed in a tube, and 1.5 mL of Chloramine-T reagent was added; the mixture was allowed to react for 25 min. Upon completion of that time, chromogenic reagent (15 g of p-dimethyl-amino-benzaldehyde, 60 mL of n-propanol, 26 mL of 70% perchloric acid were made up to a volume of 100 mL with distilled water) was added, and the tubes were introduced into a water bath at 60°C for 15 min. Samples were left to cool to room temperature; and after, absorbance was read at 550 nm in a Beckman UV-VIS spectrophotometer (Beckman-Coulter, Brea, CA, USA).

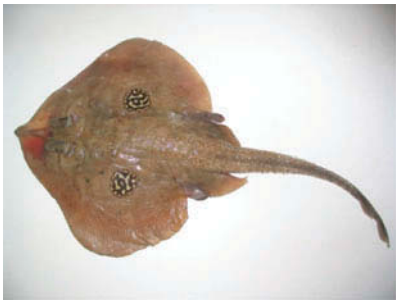
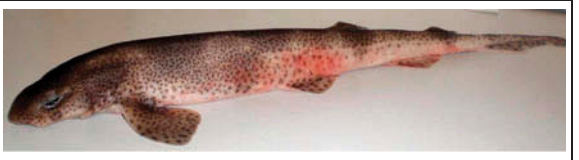
Key	Species	
CMON	*Chimaera monstrosa*	
LNAE	*Leucoraja naevus*	
NAEQ	*Nezumia aequalis*	
ETMO	*Etmopterus spp.*	
GAL	*Galeus spp*	
SCAN	*Scyliorhinus canicula*	

Figure 1. Species analyzed in this study, scientific name and keys used.

SDS-PAGE

Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by adding 100 μL of Laemmli sample buffer (1% dithiothreitol was used instead of 2-ME) to 50 μg of protein and heating for 4 min at 100°C (Laemmli, 1970). An aliquot (10 μL) of this mixture was applied to each well in 7% polyacrylamide separating gels. Gels (100 × 750 × 0.75 mm^3) were prepared according to the procedure of Laemmli (1970) and were subjected to electrophoresis at 15 mA using a Mini-Protean II Cell (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, the gels were stained with 0.04% Coomassie Blue in 25% v/v ethanol and 8% v/v acetic acid for 30 min at 60°C. Excess stain was removed with several washes of destaining solvent (25% v/v ethanol, 8% v/v acetic acid).

Molecular weights of subunits of acid soluble collagen (ASC) were estimated using molecular weight standards from BIO-RAD SDS-PAGE standards high range: Myosin (200 kDa); β-Galactosidase (116 kDa); phosphorylase B(97 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa); and analyzing the gel with the software Quantity One from BIO-RAD.

Amino acid analysis

Amino acids were analyzed in ASC after acid hydrolysis using 6 N HCl, containing 0.1% w/v phenol under inert atmosphere and heating up to 110°C for 24 h. After the hydrolysis, HCl was removed from the ASC hydrolyzate with vacuum (Moore and Stein, 1949, 1958).

The hydrolyzate was resuspended in 20 to 50 μL of 0.2 M sodium citrate buffer pH 2.2, to which a known amount of norleucine was added as an internal standard and applied to an automated amino acid analyzer (Biochrom 30 series Amino Acid Analyzer, Cambridge, UK).

Peptide maps

Peptide mapping was determined according to the methodology of Nagai et al. (2004) with some modifications. ASC collagen solubilized (10 mg/mL) in 0.5 M acetic acid and 1 M Tris pH 8.0 were diluted up to 3 mg/mL with 0.2 M Tris pH 8.0 containing 0.5% w/v SDS, heated for 5 min at 100°C and cooled. Then, 500 μg of trypsin (TPCK treated trypsin from Sigma) or 75 μg of endoprotease Glu-C from *Staphylococcus aureus* V8 (Sigma) were added, and digestion was carried out at 37°C for 6 h, in the case of trypsin and 3 h in the case of endoprotease Glu-C. After digestion, 200 μL of digested collagen were mixed with 200 μL of Laemmli sample buffer and heated at 100°C for 4 min. Collagen peptides were separated in 7% polyacrylamide gels. The standard protein markers used to estimate molecular weights were Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhidrase (30 kDa), Trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa; Amersham LMW calibration kit for SDS electrophoresis; GE Healthcare, Little Chalfont, UK). Staining was performed using PAGE silver staining kit from Fermentas (Pittsburgh, PA, USA).

Differential scanning calorimetry (DSC) analysis

Freeze-dried samples were solubilized in 50 mM acetic acid (about 5 mg of freeze-dried sample/mL). Thermoestability of ASC solutions were measured in a microcalorimeter DSC III from Setaram (France). All samples were introduced in the calorimeter at 278.15°K and left for 1 h to stabilize. After, temperature increase was set to 1°K/min up to 325°K. Two parameters were determined: the maximum denaturation temperature and transition temperature by the onset method.

Statistics analysis

Characterization of skins was performed using skin of different individuals (up to three). Triplicates were made in analytical determinations. Differences in ASC and hydroxyproline were tested by one-way analysis of variance (ANOVA), with "species" as main factor. It was applied a posteriori least significant difference (LSD) test. Significance levels were set at $p < 0.05$. Statistical tests were performed with IBM SPSS 21.0 (IBM Corp., Armonk, NY, USA).

Results and discussion

Proximate analysis

Table 1 shows moisture, protein content, and acid soluble collagen of the studied species. The average moisture and protein content of these species was 66.24 and 30.27% (N × 6.25), respectively.

Extraction of collagen from skin of fish species

The objective of the present work was the extraction and characterization of collagen of some discarded fishes, which, up to our knowledge, has never been reported before. Acid extraction of collagen is often used for obtaining collagen from fish (Karim and Bhat, 2009). This approach is widely used since the methodology can be easily applied in an industrial extraction of collagen.

The results obtained for the ASC content of the different discarded species were analyzed. The acid soluble collagen varied from 5 to 29% in dry weight basis.

Skin from the analyzed fish species differed, depending on the type of fish—i.e., skin from Ostheoichthyes, such as *Nezumia*, is rather soft; while skin from Chondrichthyes, such as *Galeus* or *Scyliorhinus*, is hard and difficult to cut. It has been described that the amount of extracted collagen reflects the amount of collagen fibers that each type of skin would have; usually harder skins show higher proportion of collagen fibers. In this case only, *Etmopterus* spp. shows a significantly lower ASC value. This shark is a deep water organism (Ebert and Stehmann, 2013) presenting a bioluminescence photogenic system located in the epidermis of the animal (Meyer and Seegers, 2012), which could make the difference in extraction efficiency in this case. Alternatively, extraction efficiency could be different because of the differences in secondary structure of the collagen: A high degree of crosslinking via covalent bonds could lead to lower extraction efficiency (Foegeding et al., 1996a). Besides, LNAE shows the highest ASC content with almost 30% of ASC (Table 1). Other authors have reported contents of collagen for fish skin similar to those obtained here. The elasmobranch *Raja kenojei* was reported to have 35.6% of ASC (Hwang et al., 2007); however, higher contents have been reported for carp and cod (41.3 and 42.5%, respectively) by Duan et al. (2009).

The acid soluble collagen provides an idea of the amount of collagen that could be present in the skin or body part of a particular species; however, it is not a quantitative extraction of collagen since there is evidence that part of the collagen cannot be extracted using this simple procedure and other agents should be employed in order to fully extract collagen, such as proteases like pepsin (Senaratne et al., 2006).

Hydroxyproline (HPro) content of skin of fish discards

HPro is often used as a method to quantify the amount of collagen in a particular tissue. Collagen contains specifically HPro, and the ratio of HPro per collagen molecule in different collagen types is known. Therefore, by knowing the amount of this amino acid, the amount of collagen can be extrapolated (it has been used a conversion factor of 12.5; Edwards and O'Brien, 1980). In order to better evaluate the content of collagen in the fish skins analyzed, the amount of HPro was determined. Contents of collagen calculated from HPro were higher than those determined from ASC extracts, as expected (Table 2). The ratio of ASC extracted, referred to the HPro determined collagen, varied with the species. In the case of *Etmopterus* spp., ASC represented as low as 20% of HPro determined collagen; however, the ASC obtained from ray (*Leucoraja naevus*) was 100% of that determined by HPro. Species-specific differences in ASC extraction may represent differences in crosslinking degree among collagen molecules at the telopeptide region, as has been previously suggested (Uriarte-Montoya et al., 2010; Zhang et al., 2009).

Table 2. Estimation of collagen from hydroxyproline content in fish skin.

Species	% Collagen (wet basis)	% Collagen (dry basis)
Nezumia aequalis (2)	14.73 ± 1.85	36.47 ± 2.85 a
Chimaera monstrosa (2)	7.85 ± 0.72	31.03 ± 1.62 a,c
Etmopterus spp. (3)	8.60 ± 1.27	27.76 ± 0.43 b,c,d
Galeus spp. (3)	14.17 ± 1.73	37.10 ± 1.39 a
Scyliorhinus canicula (3)	11.60 ± 2.10	31. 317 ± 3.48 a,d
Leucoraja naevus (2)	7.85 ± 0.05	27.43 ± 0.40 b,c,d

Numbers in parentheses represent the number of individuals used for the study. ANOVA analysis was performed for hydroxyproline content showing significant differences among species ($F = 3.626$, $p < 0.05$). Same letter indicates no statistical difference among means ($p < 0.05$).

Amino acid composition of ASC

Table 3 shows amino acid composition of the ASC of species studied and also that from calf skin collagen (data from Zhang et al., 2009). Amino acid composition has not been reported previously for the collagen of these species. Glycine content is highest in all cases, since glycine is the every third residue amino acid (Gly-X-Y) in collagen molecules, except in the telopeptide regions (Horng et al., 2007); values range in all fish species studied from 318 up to 338 Gly residues/1,000. It has been established that glycine is a key amino acid in maintaining the alpha-helix structure of the collagen molecule since absence of a side chain permits almost a free rotation capacity (Horng et al., 2007). Positions X and Y of the repeated amino acid sequence of collagen are often occupied by hydroxyproline and proline, so frequently the sum of both (imino acid content) is often taken into consideration to explain some collagen properties (Jongjareonrak et al., 2010). However, it has been reported that hydroxyproline is responsible and can play a more important role in the stabilization of the collagen helix (De Simone et al., 2008). Imino acid content is similar in all fish species studied ranging from 151 up to 177. These values are similar to those presented by cold-water fish and lower than those of warm-water fish (Regenstein and Zhou, 2006). Calf skin shows a higher value of 215 imino acid residues/1,000. The percentage of hydroxylation of proline shows

Table 3. Amino acid composition of discarded fish skin ASC (residues/1,000).

Amino acid	NAEQ	CMON	ETMO	GAL	SCAN	LNAE	CALF*
Alanine	122	91	127	114	100	99	119
Arginine	48	45	48	49	50	49	50
Aspartic a./asparagine	46	44	37	40	43	42	45
Cysteine	13	10	27	12	1	1	0
Glutamic a./glutamine	68	67	64	66	68	73	75
Glycine	326	349	318	337	338	334	330
Histidine	6	6	11	9	10	10	5
Isoleucine	11	21	18	14	13	17	11
Leucine	20	26	27	25	22	23	23
Lysine	25	25	25	26	27	27	26
Hydroxylysine	5	7	6	4	6	6	7
Methionine	19	16	22	17	14	14	6
Phenylalanine	14	15	16	14	15	14	3
Hydroxyproline	81	92	68	81	87	72	94
Proline	90	76	83	85	90	87	121
Serine	57	53	51	52	59	66	33
Threonine	26	27	20	25	27	32	18
Tyrosine	3	6	4	3	3	4	3
Valine	20	25	29	26	27	27	21
Imino acids	171	168	151	166	177	159	215
% of hydroxylation of Pro	47%	55%	45%	48%	49%	45%	44%

ASC = acid soluble collagen; NAEQ = *Nezumia aequalis* (common Atlantic grenadier); CMON = *Chimaera monstrosa* (rabbit fish); ETMO = *Etmopterus* spp. (lantern shark); GAL = *Galeus* spp. (catshark); SCAN = *Scyliorhynus canicula* (small-spotted catshark); LNAE = *Leucoraja naevus* (cuckoo ray); CALF = calf skin collagen.
*Data from Zhang et al. (2009).

some differences; although this has not been statistically confirmed. There are some species that presented higher values—such as GAL, CMON, and SCAN (between 49 and 55%); calf skin collagen presented about 45% hydroxylation of proline.

SDS-PAGE patterns of ASC collagen from discarded species

Fish skin collagen has been reported previously to comprise two or more different chains (hetero-trimers): typically two alpha 1, one alpha 2 chains, and a beta component structure that is recognized as type I collagen (Gelse et al., 2003; Bae et al., 2008; Sing et al., 2011). Most of the species studied show a type I collagen: two alpha chains of around 100 kDa and a beta component of about 200 kDa; the exception is the fish CMON whose ASC pattern showed to be different from the others (Figure 2). In this gel, alpha 1 and alpha 2 bands can be observed in most of the species. Also, beta and gamma components, dimers and trimers, respectively, can be seen in most of them. In the case of CMON, the beta component is very weak, and the alpha 2 component is hardly seen; there are two bands with molecular weight in between alpha 1 and beta dimmer (136 and 161 kDa, respectively). Also, the relative amount of alpha 2 is much lower than alpha 1; in this case, almost nonexistent. It has been described previously that an alpha 3 component may be present in fish skin (Nagai and Suzuki, 2002); however, this component would comigrate with the alpha 1 (Shigeru and Masataka, 1999). In some of the species, bands below 100 kDa have been observed (LNAE, SCAN, ETMO, GAL, and CMON); whereas, in NAEQ these bands have not been observed. These can be the product of collagen degradation, indicating that during ASC extraction some of them may be more susceptible to hydrolysis. Table 4 shows molecular weights for each main component. A clear resemblance of the component pattern and molecular weight of ASC from fish skin and collagen type I from calf skin can be observed. Although there is a slightly lower molecular weight of Chondrichthyes components, it can be said that no major differences were observed in this parameter. Teleost, like NAEQ, show a clear alpha 1 subunit, which is

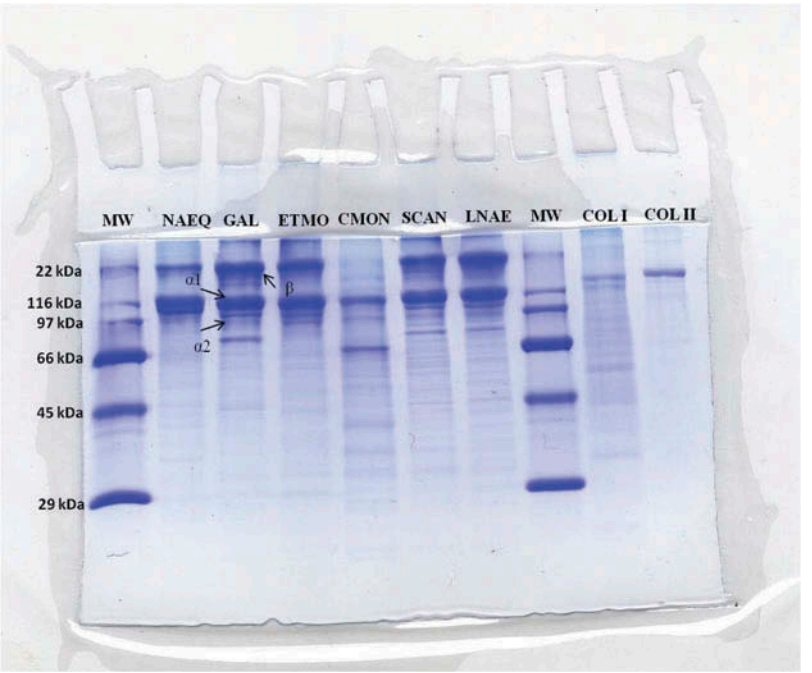


Figure 2. 7% SDS-PAGE showing ASC from different species. M.W: molecular weight standards; Col I: standard collagen type I from mammal; Col II: standard collagen type II from mammal. Keys for species are those shown in Figure 1.

Table 4. Molecular weight distribution of ASC from different species, estimated from SDS-PAGE mobilities.

Band	Col I	Col II	NAEQ	CMON	ETMO	GAL	SCAN	LNAE
Beta	206		207	204	203	207/199	210	209
Alpha 1	141	138	137	132	119	120	126	133
Alpha 2	119	102	113	111	109	97	112	112

ASC = acid soluble collagen; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Col I = collagen type I; Col II = collagen type II; NAEQ = *Nezumia aequalis* (common Atlantic grenadier); CMON = *Chimaera monstrosa* (rabbit fish); ETMO = *Etmopterus* spp. (lantern shark); GAL = *Galeus* spp. (catshark); SCAN = *Scyliorhynus canicula* (small-spotted catshark); LNAE = *Leucoraja naevus* (cuckoo ray).

the main component, but the alpha 2 component is also quite strong; whereas, in the case of the three sharks and the LNAE, the alpha 2 component is minor.

Peptide maps

Tryptic and endoprotease Glu-C digestions were performed with ASC extracted from the different skins. Figure 3A shows the tryptic digests separated by SDS-PAGE. All ASC high molecular weight components were markedly digested by trypsin, except for SCAN and CMON, which showed some bands of different size (lower than 66 kDa).

Trypsin cleaves peptide chains at the carboxyl side of the amino acids Lys or Arg. In general, collagens from marine organisms are usually hydrolyzed in several sites, and no bands are seen in SDS-GELS, since fragment size are lower than 20–30 kDa (Zhang et al., 2009). From these results, it appears that SCAN and CMON ASC are more resistant to trypsin hydrolysis than the other ASC. Since content of Arg and Lys are very similar in all these species, different positions of those amino acids are along the protein chains.

Figure 3B shows the endoprotease Glu-C digestions of the ASC separated by SDS-PAGE. In general, susceptibility to digestion was lower in all cases, reflecting the relatively low presence of glutamic residues in the collagen subunits. However, it can be noticed that in all cases, alpha and beta components were markedly hydrolyzed, and the differences observed can be attributed to differential primary structure in different species.

DSC analysis

Analyses of DSC were performed with ASC solubilized in 50 mM acetic acid. As can be seen in Table 5, all ASC from the species analyzed have lower denaturation temperature than calf skin collagen, between 8 to 15.2°C, as has been previously described for other fish and mammals (Hwang et al., 2007). Besides, the differences among these species were of 7.2°C. Denaturation temperatures of *Etmopterus* spp. and *Galeus* spp. are very similar to those reported for skate skin (*Raja kenojei*; Hwang et al., 2007), which also belongs to class Chondrichthyes. Furthermore, *Leucoraja naevus* (LNAE) has a higher denaturation temperature than skate skin, but it is lower than eagle ray, red stingray, or yantai stingray (Hwang et al., 2007; Bae et al., 2008). It is noteworthy that the denaturation temperature of *Chimaera monstrosa* is higher in comparison to the other species studied and also compared to other species of Chondrichthyes found in literature (Hwang et al., 2007; Bae et al., 2008).

It has been described that a lower thermal denaturation temperature is related to lower degree of proline hydroxylation in fish collagens (Kimura et al., 1988) and lower imino acid content (Foegeding et al., 1996b). Our results show that there is a positive correlation between the hydroxyproline content and the temperature of denaturation ($R^2 = 0.7537$) and also between the content of imino acids and denaturation temperature ($R^2 = 0.8797$).

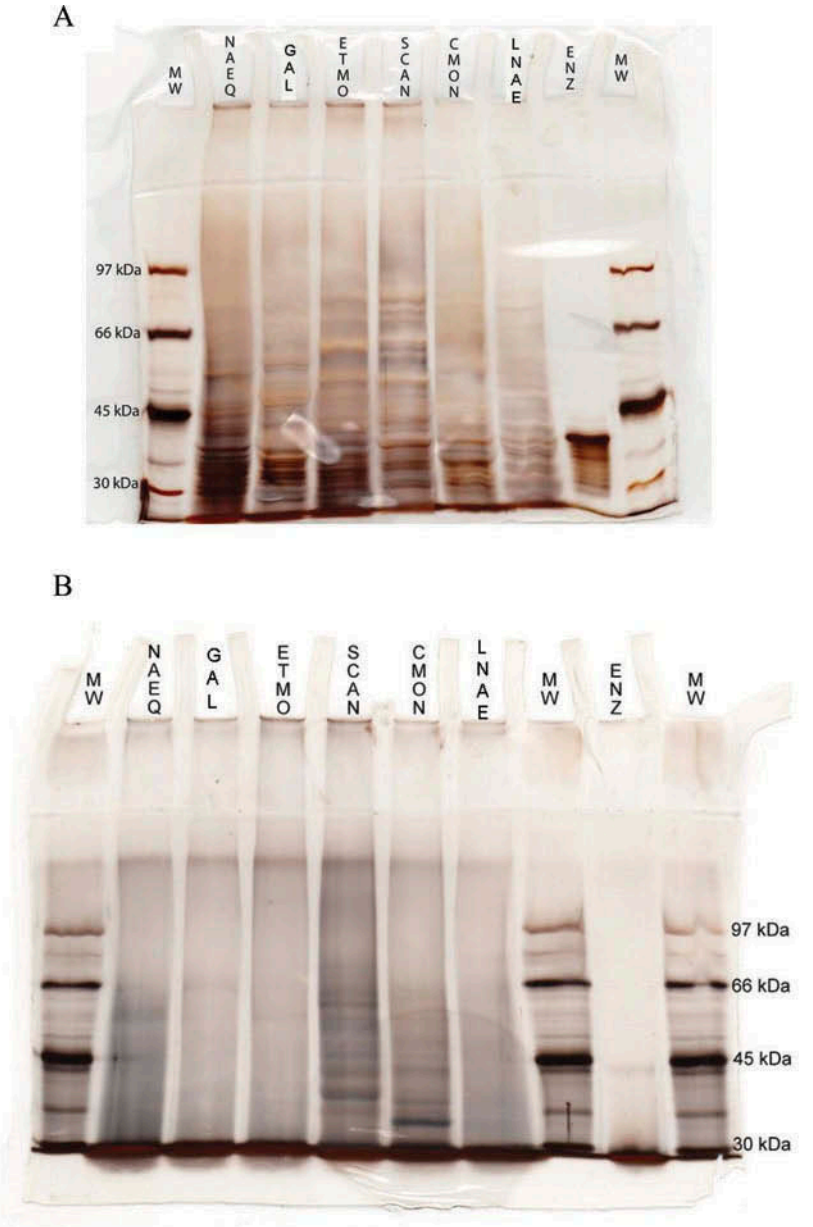


Figure 3. Gel A: ASC from different species digested with trypsin. Gel B: ASC from different species digested with Endoprotease Glu-C.

Conclusions

Acid soluble collagen from the skin of six fish species was analyzed. Five of these species were Chondrichthyes and one teleost. Extracted ASC from the fish skin was about 20% on a dry basis. Amino acid composition revealed the typical composition of collagen, with very few differences among species. SDS-PAGE analysis of ASC suggests the classification as type I, with the profile of CMON being very characteristic and different from those of the other studied species. Specific profiles were obtained after protease digestion, especially in the case of the digestion with

Table 5. Temperature of denaturation, determined by DSC, of ASC from calf skin collagen and the six fish species studied.

Species	T_d^m (°C)
NAEQ	31.55
CMON	35.65
ETMO	28.55
GAL	28.45
SCAN	33.15
LNAE	30.25
Calf skin collagen SIGMA	43.65

DSC = differential scanning calorimetry; ASC = acid soluble collagen; NAEQ = *Nezumia aequalis* (common Atlantic grenadier); CMON = *Chimaera monstrosa* (rabbit fish); ETMO = *Etmopterus* spp. (lantern shark); GAL = *Galeus* spp. (catshark); SCAN = *Scyliorhynus canicula* (small-spotted catshark); LNAE = *Leucoraja naevus* (cuckoo ray).

endoprotease Glu-C. Denaturation temperature of the studied ASC correlated with the imino content and HyPro content of the ASC.

As far as the authors know, this is the first article reporting data on skin collagen of *Nezumia equalis, Chimera monstrosa, Etmopterus* spp., *Galeus* spp., *Scyliorhinus canicula*, and *Leucoraja naevus*. Since collagen from the species studied is now described and the main chemical characteristics are similar to those described for other fish, the authors can affirm that skin from those highly discarded fish species is an alternative of mammalian sources and therefore is useful in obtaining collagen for different industrial applications.

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