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Assessment of the denaturation of collagen protein concentrates using different techniques

Abstract: The use of collagen and gelatin in the field of regenerative medicine is widely extended. However, most of the studies in this topic are focused on the scaffolds' properties, but only a few are related to the properties of the raw material used. The raw material analysis not only consists of a study of the composition, but also of the denaturation degree that can influence the processing and properties of the structure of the scaffold. Thus, the denaturation degree analysis of different collagen proteins was performed and assessed by the comparison of four different methods: differential scanning calorimetry (DSC), Fourier transform Infrared Spectroscopy (FTIR) and circular dichroism (CD) spectra and sulfhydryls content analysis. DSC measurements put forward a glass transition between 88°C and 95°C as well as from the FTIR measurements; the characteristic peaks for proteins are evidenced. However, from the sulfur content, only a small proportion of free sulfhydryls are present with respect to their total amount. In addition, CD spectra allow to estimate the secondary structure of the protein by the analysis of the α-helix and β-strand and also quantify the denaturation degree with the 'positive/negative ratio' (RPN) from the CD profiles, obtaining values in the range between 25% and 100%.

Keywords: circular dichroism; collagen; denaturation degree; DSC; FTIR.

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Introduction

Currently, most studies in regenerative medicine focus on the development of two- and three-dimensional structures to act as promising scaffolds for tissue engineering, due to the increasing importance of tissue engineering in the recovery and care of diseases (Langer and Vacanti, 2016). However, the efficiency of the process is quite linked to the properties of the scaffold. For that reason, research in this topic is focused on the properties of the three-dimensional structures fabricated (mechanical, morphological or biological properties) (Ritzoulis et al., 2005). As a matter of fact, these properties are closely related to the raw material used for the fabrication of the scaffold. The scaffolds can be produced from either synthetic or natural polymers, depending on the properties to be promoted (synthetic for better mechanical properties or natural for a better biocompatibility) (Aldana and Abraham, 2017; Deng et al., 2018).

Collagen protein is a good example, as it is one of the most used polymers for obtaining scaffolds (Jose et al., 2009; Correia et al., 2013; Sionkowska and Grabska, 2017). Collagen is present in animal tissues, being more than the 30% of their total protein content. It is based on a triple helix of three polymeric chains of *ca.* 1000 amino acids connected by peptide bonds (Jilek and Helmut, 1978; Luparello and Sirchia, 2011; Merrett et al., 2012). Despite the good biological properties of the resulting scaffolds using collagen, one of the drawbacks is related to the poor mechanical properties of those scaffolds (Chan et al., 2014; Davidenko et al., 2015). For that reason, additional reinforcement is necessary to improve their mechanical properties. This reinforcement can be achieved with the use of a chemical crosslinking agent such as glutaraldehyde, which is one of the most used agents in tissue engineering (Ricks et al., 1998; Oryan et al., 2018), an enzymatic crosslinking agent such as transglutaminase (Orban et al., 2004) or by a physical reinforcement through a heating process or ultraviolet treatment (Perez-Puyana et al., 2016; Zhang et al., 2016).

Collagen-based scaffolds may be constructed either in the native form or the denatured form (gelatin). Gelatin,

the natural polymer obtained from collagen by acidic or alkaline processing and thermal denaturation, is widely used as a scaffold material because of its advantages in tissue engineering applications (Chen et al., 2016; Aldana and Abraham, 2017). It is characterized by its high biocompatibility, bioresorbability, non-immunogenicity, and its favorable functional properties. Studies reveal that gelatin does not elicit any noticeable antigenic responses upon implantation and promotes cellular growth (Allam et al., 2012). Its chemical and physical properties are similar to those of collagen, but with even better biological responses (Echave et al., 2017).

Thereby, it is important to consider the properties of the raw material used in the scaffold fabrication and, specifically for collagen studies, its denaturation degree. Thus, a characterization of the raw material is advisable in order to make a good selection. The determination of the denaturation of proteins implies the study of their secondary structure. Thus, circular dichroism (CD) or Fourier transform infrared spectroscopy (FTIR) are widely used techniques for this purpose (Goormaghtigh et al., 2009), but they are not the only ones, because others such as microthermal analyses or atomic force microscopy can be performed (Lin and Goh, 2002). However, the precise mechanism of collagen denaturation remains unknown due to its crosslinking and polymeric nature (Ravikumar and Hwang, 2008). CD measurements applied to biological systems give information about their conformational changes or the folding and unfolding of proteins (Kelly and Price, 2000). The advances in this field have allowed the analysis of the spectra obtained using specific software. K2D software, for example, gives an estimation of the percentages of secondary structure in proteins (Orrego Cardozo et al., 2015). FTIR, in turn, gives information about the chemical bonds present in the molecules studied. When analyzing biological molecules (such as proteins), the carboxyl and amino bonds are the most interesting groups to consider, since the intensity of these peaks gives information about the denaturation of the protein studied (Muyonga et al., 2004a,b).

Furthermore, other possible techniques to be used are free and total sulfhydryls or differential scanning calorimetry (DSC) measurements. The former allows the determination of S-S bonds, which could give an idea of the agglomeration of the protein and, for that matter, of its denaturation (Greensteik, 1938). Meanwhile, DSC measurements study the thermal properties of the protein, which vary depending on its denaturation (showing specific peaks depending on whether it is collagen or gelatin) (Beveridge et al., 1974; Badea et al., 2012).

Thus, the aim of this work was to assess three different collagen proteins and compare their denaturation degree using a combination of four different techniques (free and total sulfhydryls, DSC, FTIR and CD). The information obtained in this study can be useful for the selection of a suitable raw material for the fabrication of scaffolds in further research.

Results and discussion

Characterization of protein concentrates

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been performed for the different protein concentrates. Figure 1 shows the profile obtained as well as a standard pattern with different molecular weights. As may be observed, two different profiles can be clearly distinguished. Meanwhile, lanes 1–3 show the profiles for pork HI95, turkey and pork T95, respectively. The profiles obtained are very similar, mainly formed by two α-chains, corroborating their collagen nature because it has been proven that the major components of type I collagen are composed of two α-chains (α_1- and α_2-chains) (Chi et al., 2014). The profile shown in lane 4 corresponds to fish protein. This profile is different from the one explained before. In this sample, α- and β-chains are its major constituents. Comparing this profile with the ones obtained in other studies, as the ones carried out by Kittiphattanabawon et al. (2015), in which α- and β-chains were found as the dominant constituents in gelatin protein, it can be confirmed that fish protein is pure gelatin protein.
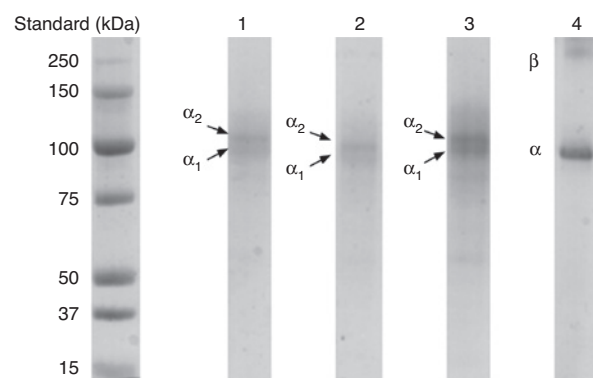

Figure 1: SDS-PAGE of the different protein concentrates: pork HI95 (lane 1), turkey (lane 2), pork T95 (lane 3) and fish (lane 4). Standard molecular weight values has also been included.

Free and total sulfhydryls

The values obtained for the total and free sulfhydryls for the four collagen protein concentrates are shown in Table 1. The values obtained from both free and total sulfhydryls provide information about those sulfhydryls forming disulfide bonds (S-S). Although it is true that the number of free sulfhydryl groups is not necessarily a measure of denaturation, it is a good indicator of the aggregation of a protein (Margaritis et al., 2011). A gelatin protein is defined by a low number of free sulfhydryls, as it can be seen in the results obtained for fish gelatin protein. The other proteic samples presented higher values for the free sulfhydryls. As for the total sulfhydryls, although the same tendency applies, the only significant difference was found again for the lowest values of the fish gelatin protein.

It is important to point out that with more sulfhydryls present, more disulfide bonds can be generated. However, according to the results, it can be asserted that the low presence of free sulfhydryls in all the protein concentrates studied (compared with the total sulfhydryls content) indicates that these proteins have a certain oxidation (probably due to the high temperatures reached during their production process).

Differential scanning calorimetry

The heat flow patterns obtained from DSC measurements in a temperature range between $-20°C$ and $140°C$ for the four studied protein concentrates are shown in Figure 2. A first run (dash line) and a second run (straight line) were conducted to detect irreversible dips. Two different shapes can be observed: on the one hand, pork (HI95 and T95) and turkey collagen concentrates exhibited a similar profile (Figure 2A–C, respectively). These profiles showed three main dips (T_1, T_2 and T_3) and an inflection point (T_4). A remarkable dip (T_1) was found at *ca.* $0°C$, related to ice melting (Kopp et al., 1990). The second dip (T_2), appearing in the range of $25°C–35°C$ ($25°C$, $33°C$ and $35°C$ for turkey, pork HI95 and pork T95, respectively), is characteristic of partial gelatinized (denatured) collagen (Beveridge et al., 1974), which indicates that these three collagen concentrates present a certain denaturation degree. Furthermore, the three samples showed an endothermic peak in the region between $60°C$ and $63°C$, which is reversible, since it did not appear in the re-scanning (2nd run). The intensity of this reversible endothermic peak is low, and it is probably related to the physical ageing of these collagen concentrates (Farahnaky et al., 2008). In addition, the inflection point (T_4), observed in the range between $95°C$ and $100°C$, corresponds to the glass transition temperature, which is the temperature at which many changes in the properties (enthalpy, viscoelasticity etc.) of materials take place (Urbaniak, 2011).

On the other hand, the profile obtained for the fish protein concentrate (Figure 2D) only showed two important signals: a dip at $71°C$ (T_3), related to the physical ageing of the protein concentrate, and an inflection point at $88°C$ (T_4), which correspond to the glass transition of this protein. The T_g of the fish protein concentrate appears at $88°C$ in the second run because the gelatin molecules could undergo a better interaction after the first run, obtaining a stiffer matrix and a displacement of the T_g value (Tongnuanchan et al., 2016). In addition, the peak related to ice melting did not appear due to the low moisture of this protein concentrate (below 1%).

Fourier transform infrared spectroscopy

The FTIR spectra of pork HI95, pork T95 and turkey protein concentrates (Figure 3A–C, respectively) are shown in

Table 1: Free/total sulfhydryl values and ratio, RPN values, approximated denaturation degree and estimation of the secondary structure (α-helix and β-strand) for the four different collagen protein concentrates (pork HI95, turkey, pork T95 and fish).

Raw material	Sulfhydryls (µmol/g)			RPN	Denaturation degree (%)	Secondary structure	
Protein system	Free	Total	Free/total ratio (%)			α-Helix	β-Strand
Pork HI95	45 ± 15[a]	1658 ± 343[a]	2.7	−0.028	25	8.8 ± 0.9[a]	27.1 ± 4.2[A]
Turkey	108 ± 30[b]	1819 ± 171[a]	5.9	0.005	65	7.4 ± 1.0[a]	30.5 ± 2.5[A]
Pork T95	155 ± 35[b]	2300 ± 450[a]	6.7	0.015	75	5.5 ± 1.1[b]	31.0 ± 2.0[A]
Fish	49 ± 6[a]	645 ± 186[b]	7.6	0.037	100	2.9 ± 0.3[c]	44.9 ± 6.2[B]

Values with different letters are significantly different ($p < 0.05$).
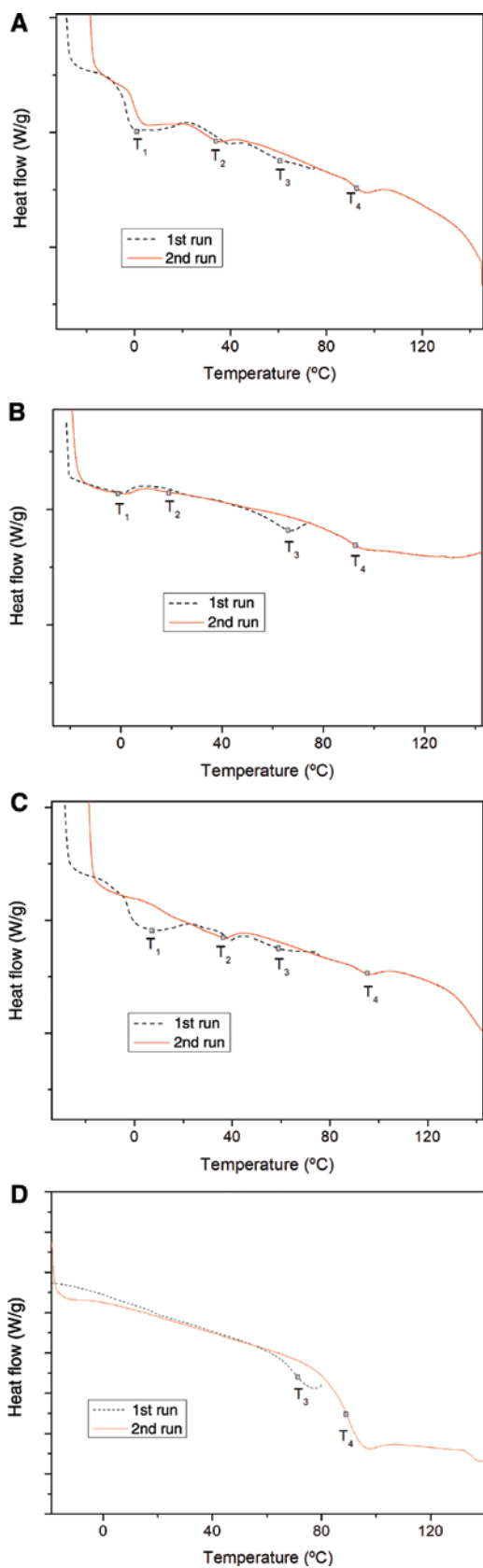
Figure 2: DSC profiles of the four different protein concentrates: (A) pork HI95, (B) turkey, (C) pork T95 and (D) fish.

Figure 3. In addition, the profile obtained for fish gelatin protein was also included (Figure 3D). Moreover, the main peaks and their assignments can be seen in Table 2. All of them present the same profile, with six characteristic signals. The first signal is located at 3300–3200 cm^{-1}, and it corresponds to N-H stretching. Then, there are two signals in the range between 2950 and 2850 cm^{-1}, characteristic of symmetrical and asymmetrical CH$_2$ stretching. Subsequently, the other three signals are located at *ca.* 1635 and 1525 cm^{-1} (C=O stretching) and 1240 cm^{-1} (N-H bending). It is important to highlight the peaks referred to N-H bending and C=O, N-H and C-N stretching, which are the characteristic peaks for proteins. However, the four spectra present slight differences, indicating some variances in the structures of these proteins (Muyonga et al., 2004a,b). These differences are referred to the intensity of the bands, a fact related to prominent absorption bands associated directly with the secondary structure of proteins found in gelatins (Cebi et al., 2016).

Apart from the intensity of the bands (sharper and stronger peaks), the study of the amide I signal allows a better differentiation between collagen and gelatin. This fact was reported by Payne and Veis (1988), showing that this peak is a combination between two marked peaks at *ca.* 1650 and 1633 cm^{-1}, and depending on the peak with the highest intensity, the sample can be considered as collagen or gelatin. Thus, a zoom in of this signal was included (Figure 4). As can be seen in Figure 4, the signal for the fish gelatin protein shows two marked peak at 1633 and 1650 cm^{-1} (characteristic of gelatin proteins), whereas the other three samples (pork T95, pork HI95 and turkey protein concentrates) do not show a discrete peak, but a broad peak as a combination of both. For that reason, it can be deduced that these three protein concentrates present a certain denaturation degree, so they are not either native collagen or gelatin, as the fish protein.

Circular dichroism

The CD spectroscopy of the four protein samples at the wavelength range of 190–240 nm is shown in Figure 5. The CD spectra are very similar for the four protein systems, showing a minimum at *ca.* 198 nm and a maximum at 222 nm. Two different studies could be assessed from the CD spectra obtained for the four protein concentrates studied. First, according to Gopinath et al. (2017), there is a relationship between the maximum (positive) and minimum (negative) peaks and the denaturation of the protein (RPN). According to the assumption that a higher
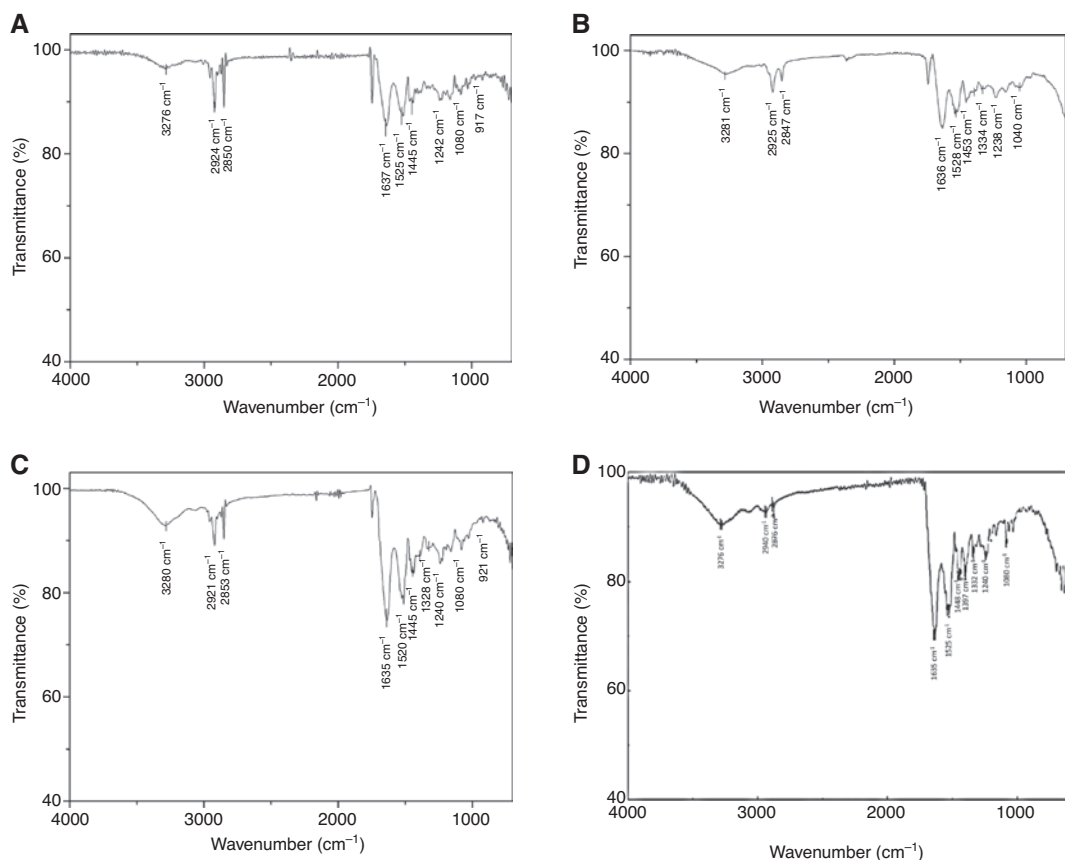
Figure 3: FTIR spectra of the four different protein concentrates: (A) pork HI95, (B) turkey, (C) pork T95 and (D) fish.

Table 2: FTIR spectra peak position and assignments for four different protein concentrates (pork HI95, pork T95, turkey and fish).

Signal	Region			Peak (cm⁻¹)		Assignments	Reference
		Pork HI95	**Turkey**	**Pork T95**	**Fish**		
A	Amide A	3276	3281	3280	3276	N-H stretch	[24]
B	Amide B	2924	2925	2921	2940	CH_2 asymmetrical stretch	[28]
C		2850	2847	2853	2876	CH_2 symmetrical stretch	[28]
D	Amide I	1637	1636	1635	1635	C=O stretch	[32]
E	Amide II	1525	1528	1520	1525	N-H bend coupled with C-N stretch	[30]
F		1445	1453	1445	1448	CH_2 bend	[33]
G		1380	1393, 1334	1400, 1328	1397, 1332	CH_2 wagging of proline	[28]
H	Amide III	1242	1238	1240	1240	n-h bend	[30]
I		1080, 917	1040	1080, 921	1080, 920	Skeletal stretch	[34]

ratio implies a more denatured protein and considering the CD profiles shown in Figure 5, the most denatured protein is the fish gelatin protein (in accordance with the FTIR measurements) and the lowest one is the pork HI95 protein concentrate.

In addition, an approximation to the denaturation degree can also be obtained from the CD spectra, since a higher RPN ratio implies a more denatured protein, so a more negative peak corresponds to a lower denaturation. The RPN value for 100% native collagen structure is *ca*. −0.10 (Gopinath et al., 2017), and the RPN value obtained for the fish gelatin can be associated to a 100% denatured collagen; hence, with these values, it is possible to give a qualitative approximation of the denaturation degree of the other three protein concentrates studied (pork HI95, pork T95 and turkey) (Table 1) and
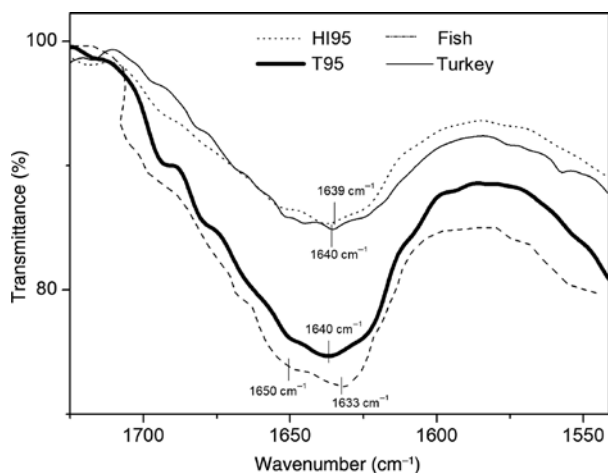
Figure 4: Close up of the amide I band from the FTIR spectra of the four different protein concentrates: pork HI95, turkey, pork T95 and fish.
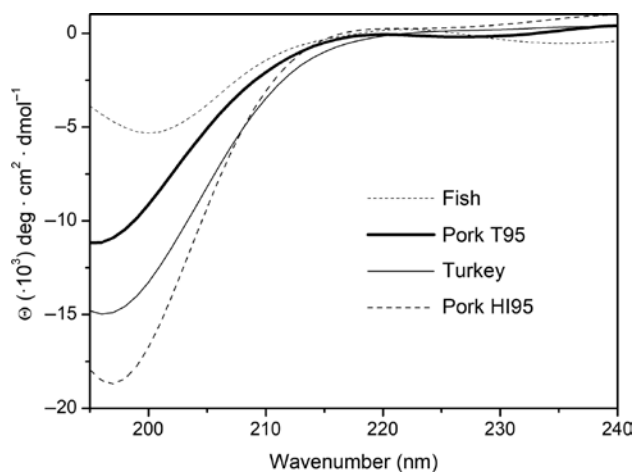

Figure 5: CD spectra of the four different protein concentrates: pork HI95, turkey, pork T95 and fish.

compare the values obtained with the values belonging to the pure and denatured collagen (shown above). The results illustrated that pork T95 had the highest RPN values, which are associated to a higher denaturation degree.

Considering the prediction of the secondary structure (Table 1), the pork HI95 protein concentrate presents the highest proportion of α-helix (8.8%) and the lowest of β-strand (27.1%), whereas the fish protein system presents the lowest proportion of α-helix (2.9%) and the highest of β-strand (44.9%). However, in general, the four protein concentrates present a significantly lower proportion of α-helix in the structure, compared to the proportion of β-strand, which is related to an increase in the

denaturation degree, in accordance to the results showed in the study (Kelly and Price, 2000).

Furthermore, according to Echave et al. (2017), the use of denatured collagen protein (gelatin protein), instead of collagen, produces better cell growth during the implantation of the scaffolds (developed using this protein as raw material). For that reason, an increase in the denaturation degree of collagen protein, used as raw material for the formation of scaffolds, favors the biological response of these scaffolds in tissue engineering applications.

Concluding remarks

The study of the different collagen protein concentrates reveals that all of them showed a certain denaturation degree. In fact, sulfhydryl content and DSC measurements also evidence that aggregation and physical aging are present in protein concentrates but have different sulfur contents. Both effects, aggregation and physical aging, are consequence of production process and storage, respectively. However, specifically, all the samples presented the characteristic FTIR profile for proteins (N-H bending, N-H C-N stretching, etc.), with fish and pork T95 standing out due to their sharper and broader bands (typical of gelatin-like proteins), which can be better seen in the deconvolution of the amide I signal. In addition, the CD spectra showed that the denaturation degree of all these protein concentrates was over 40% (fish protein was almost 100% denatured). Furthermore, the higher the denaturation degree, the higher the amount of β-strand present in the structure of the proteins studied and the better biological response of the scaffolds produced.

In general, four different techniques have been used to analyze the denaturation degree, with their advantages and drawbacks. Sulfhydryls content gives information about the aggregation of the protein rather than the denaturation, although a relation between both can be carried out. Regarding FTIR, the denaturation can be deduced based on the peaks at 1650 and 1633 cm^{-1}, but it is difficult to quantify due to the overlapping of these peaks. Considering the DSC spectra, it is interesting to study thermal peaks, which differ depending on the denaturation of the sample, although the heating process may induce additional denaturation. Finally, the CD measurements are the most suitable because it allows to estimate the secondary structure of the protein and quantify the denaturation degree with the RPN ratio.

Materials and methods

Materials

Collagen concentrates in the form of powder from two different sources were used: pork (pork HI95 and pork T95) and turkey. They were supplied by Essentia Protein Solutions (Grasten, Denmark). The composition was known for all the powders, with a protein content over 90 wt.% (except for turkey collagen protein concentrate, which had a protein content of 77 wt.%). Pure gelatin protein from fish was used as reference. It was supplied by Henan Boom Gelatin Co. Ltd. (China), and it presents a protein content higher than 95 wt.%. The complete compositions of the four samples are shown in Table 3.

The imino acid content (Pro and Hyp) for the different proteins revealed a proline and hydroxyproline content in the range of 11%–12% and 7.5%–9%, respectively, for all the collagen proteins. However, the gelatin protein presented a proline content of 7%–8% and 4%–5% of hydroxyproline. These values are in accordance with the values presented in the studies carried out by Eastoe (1955, 1957).

The other reagents used (acetic acid, urea and EDTA) were purchased from Panreac Química (Barcelona, Spain).

Characterization of protein concentrates

Four different techniques (free and total sulfhydryls, DSCy, FTIR spectroscopy and circular dichroism) were used to characterize the collagen powders from different sources.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Electrophoresis tests were performed using polyacrylamide gels (10%) in the presence of sodium dodecyl sulfate (SDS-PAGE) according to Laemmli method (1970). The molecular weights of extracted protein fractions were determined, using SDS-PAGE gels, by considering the relationship between the logarithm of protein molecular weight and electrophoretic mobility,

using as analytical standard 'Protein Plus Protein Standards' (Bio-Rad, Richmond, CA, USA). All solutions were carried out at a concentration of 10 mM, and the ionic strength of each solution was adjusted at 154 mM by adding NaCl. Finally, 2-mercaptoethanol was added to minimize chemical interactions among different protein fractions.

Free and total sulfhydryls

'Total sulfhydryls' are referred to the total of S-groups in the protein, whereas 'free sulfhydryls' are referred to the SH-groups that are not forming disulfide bonds. The determination of the concentrations of free and total sulfhydryl groups was performed using the method developed by Beveridge et al. (1974) and Thannhauser et al. (1984), respectively.

Briefly, the samples (1 mg/ml) were suspended in a buffer containing 0.086 mol/l Tris-HCl (pH 8.0), 0.09 mol/l glycine, 4 mmol/l EDTA and 8 mol/l urea. Dispersions were stirred at 25°C for 10 min at 500 rpm in a thermomixer and then centrifuged at 12,000 rpm (10 min, 10°C). The supernatant was incubated in Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid in Tris-Gly buffer, 4 g DTNB l^{-1} methanol) for free sulfhydryl groups and in 1 ml 2-nitro 5-thio-sulfobenzoic acid for total sulfhydryls. Absorbance at 412 nm was measured in a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA, USA). For the calculations, the molar extinction coefficient of 3-thio-6-nitrobenzoate (13,600 l·mol^{-1}·cm^{-1}) was used. The protein concentration of the extracts was determined by the Bradford method (Bradford, 1976). This assay is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein – the dye binds to primarily basic (especially arginine) and aromatic amino acid residues. The color change is observed measuring the absorbance at 595 nm. A standard curve of bovine serum albumin was used for the protein quantification.

Differential scanning calorimetry

DSC experiments were performed with a Q20 (TA Instruments, New Castle, DE, USA), using 3- to 8-mg samples, in hermetic aluminum pans. A heating rate of 10°C/min was selected. The sample was purged with a nitrogen flow of 50 ml/min. A first run was performed until 80°C; then, the sample was cooled and heated again to 150°C.

Table 3: Composition of the four different collagen protein concentrates (fish, turkey, pork HI95 and T95).

Composition				Raw material
	Fish	Turkey	Pork HI95	Pork T95
Protein (%)	97.9 ± 0.1	80.3 ± 0.4	94.2 ± 0.9	90.4 ± 0.2
Moisture (%)	0.4 ± 0.1	10.0 ± 0.9	3.2 ± 0.8	7.4 ± 0.3
Lipids (%)	0.3 ± 0.1	8.3 ± 1.8	0.3 ± 0.1	0.7 ± 0.1
Ash (%)	0.6 ± 0.3	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.2

Fourier transform infrared spectroscopy

The FTIR spectrum was obtained for discs containing a 2-mg collagen sample using a FT/IR-4100 spectrophotometer (Jasco, Tokyo, Japan). The spectra were obtained for a wavenumber range from 4000 to 600 cm^{-1} and processed with Jasco Spectra Manager™ software. Furthermore, the deconvolution of the amide I signal was performed for all the spectra obtained.

Circular dichroism

Electronic CD spectra were recorded in a Biologic Mos-450 spectropolarimeter. A standard quartz cell of 10-mm path length was used. Scans were taken from 190 to 240 nm under a nitrogen atmosphere. Spectra were performed at a fixed collagen concentration of 0.01 g/l for each type of protein concentrate in acetic acid (0.05 м). All CD spectra of collagen solution were solvent subtracted, and each spectrum was obtained from an average of 10 runs at a fixed temperature of $25.1 \pm 0.1°C$ with a 10-min equilibration before each scan. The spectra obtained were expressed in terms of mean residue ellipticity $(deg \cdot cm^2 \cdot dmol^{-1})$ (Orrego Cardozo et al., 2015). This term can be expressed as

$$[\theta]_{\mathrm{mrw}} = \frac{\mathrm{MRW}\theta}{10dc}, \qquad (1)$$

where θ is the observed ellipticity (degrees), d is the path length (cm) and c is the concentration (g/l).

From the CD spectra, the 'positive/negative ratio' (RPN) was calculated, using Equation 2:

$$\mathrm{RPN} = \frac{\theta_p}{\theta_n}, \qquad (2)$$

where θ_p and θ_n are the positive and negative peaks, respectively.

Furthermore, an estimation of the secondary structure content (α-helix and β-strand) of these proteins was carried out using the deconvolution program K2D2, a re-implementation of the K2D program developed by Andrade et al. (1993).

Statistical analysis

At least three replicates were carried out for each measurement. Student's *t*-test and one-way analysis of variance $(p < 0.05)$ were performed using PASW Statistics for Windows version 18 (SPSS, Chicago, IL, USA). Standard deviations were calculated for selected parameters.

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