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Molecular and structural characteristics of cod gelatin films modified with EDC and TGase

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a b s t r a c t

Cod gelatin films before and after cross-linking of gelatin with 1-ethyl-3-(3-dimethylaminopropyl) car-bodiimide (EDC) or transglutaminase (TGase) have been characterized by Fourier transform infrared (FT-IR) spectroscopy and differential scanning calorimetry (DSC) analysis. For comparison, a film pre-pared from unmodified pig gelatin has been also analysed. The difference spectra showed that cod gelatin during the film formation involved first of all water-to-amide hydrogen bonds, and the film from pig gel-atin contained water-to-amide, amide-to-amide and water-to-water hydrogen bonds. A higher number of hydrogen bonds in the structure of the film from pig gelatin contributed to much better recovering of the helical structure in this film than in the film from cod gelatin, as the peaks at about 1663 cm^{-1} in the amide I band and at about 1537 cm^{-1} in the amide II band in the second-derivative spectra revealed. The recovered helical structure, in turn, resulted in a significantly higher melting enthalpy value in the case of the film from the pig gelatin. After modification of cod gelatin with EDC or TGase, the inter-chain cross-linkages formed in the films led to the conformation of gelatin with no indications of helical ordering. An increase of melting temperature of gelatin films by 7 °C on EDC and by 10 °C on the TGase modifications was related to the formation of covalent cross-links, and a decrease of glass temperature by 28 °C and 7 °C on EDC and TGase cross-linking, respectively, demonstrated the plasticizing effect of water.

Keywords: Fish gelatin film, Cross-linking, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) Transglutaminase (TGase), FT-IR spectroscopy, DSC analysis

1. Introduction

Due to good gelling properties and hence good film-forming ability, gelatin from various sources (mammalian and fish) has been readily used to form films. In particular, a lot of research in recent times has been concentrated on the application of fish gelatin as a component of packaging films (Gómez-Guillén et al., 2009; Sztuka & Kołodziejska, 2008a, 2008b).

The properties of gelatin films are influenced by many factors, among them are those governed by their physico-chemical structure. This structure is determined by the intrinsic properties of collagen from which gelatin is obtained, the method of gelatin extraction from raw material, and the physical parameters used in the film preparation (Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009; Rahman, Al-Saidi, & Guizani, 2008).

Regardless of the animal species from which collagen is derived, it is characterized by a triple-helical structure, which requires the presence of a repeating amino acid sequence $(Gly–X–Y)_n$. In the latter, X and Y represent any amino acid, but most frequently these are imino acids, Pro and Hyp, respectively. This structure is stabilized by intra-molecular hydrogen bonds formed between the amino group of Gly of the backbone of one chain, and the carbonyl group of a residue in the X-position of the backbone of a neighboring chain (Harrington & Rao, 1970). The amino and carbonyl groups can also be connected by water molecules. Since the non-polar pyrrolidine residues form hydrophobic rather than intra-molecular hydrogen bonds, therefore Hyp, not Pro, is engaged in water-mediated hydrogen bonding (Privalov & Tiktopulo, 1970). Other stabilizing factors are due to the regular water structure near the macromolecules, and steric restrictions imposed by the pyrrolidine rings (Privalov & Tiktopulo, 1970; te Nijenhuis, 1997). Because collagen prepared from mammalian and warm water fishes contains higher amounts of Pro and Hyp than fish species from a colder temperature environment, it has a higher number of hydrogen bonds and hence its helix is better stabilized and has a higher melting temperature than the collagen prepared from low temperature fish species (Gudmunsson & Hafssteinsson, 1997; Gómez-Guillén et al., 2002; te Nijenhuis, 1997).

The conversion of collagen into gelatin, a raw material, particularly from warm-blooded animal species, requires acid or alkaline pretreatment (Stainsby, 1987). During this process the protein structure is disorganized, and subsequent heat treatment leads to

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the destabilization of the triple helix by the rupture of hydrogen and covalent bonds. Thus, although the composition of gelatin is closely similar to that of the collagen (Eastone, 1955), the gelatin molecules in the resultant solution receive the coil conformation composed of single random chains. On reducing the temperature of the solution, the polymer chains undergo a conformational coil-helix transition, during which they tend to recover the collagen triple-helical structure. The properties of gelatin films are closely related to the level at which gelatin recovers that structure, i.e., to the gelatin renaturation level, since the renatured triple helices act as junctions from which a film network is formed (Achet & He, 1995). The type of chemical pretreatment and parameters of extraction, as well as the temperature of casting, the rate of drying and the concentration of gelatin in the film-forming solution mainly influenced the level of the gelatin renaturation. Generally, in films cast at temperatures below their melting point, the gelatin macromolecules form partially collagen-like helical structure, while in films cast at temperatures above their melting, gelatin macromolecules take the conformation of a random coil with no indications of ordering. Besides, the closer the temperature of drying to the melting temperature, the higher gelatin concentration is required in order to achieve the largest possible degree of renaturation of the collagen-like helical structure (te Nijenhuis, 1997).

Similarly to other biopolymers, before gelatin can be applied to food packaging materials, usually its hydrophilicity needs to be reduced, and sometimes its poor mechanical properties must be improved; this can be achieved by physical, chemical or enzymatic modifications (Bigi, Bracci, Cojazzi, Panzavolta, & Roveri, 1998; Bigi, Cojazzi, Panzavolta, Rubini, & Roveri, 2001; Bigi et al., 2000; Cristiano, Fayad, Porto, & Soldi, 2010; de Carvalho & Grosso, 2004; Kim, Nimni, Yang, & Han, 2005).

Our previous studies have shown that chemical and enzymatic cross-linking of fish-skin gelatin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and transglutaminase (TGase) effectively decreased film solubility from 100% for unmodified sample to about 10% and 30% for modified samples with EDC and TGase, respectively (Piotrowska, Sztuka, Kołodziejska, & Dobrosielska, 2008). It is known that TGase provides cross-links of protein chains through the formation of the covalent linkages between γ-carboxyamide groups of peptide-bound glutamine residues and ε-amino groups of lysine (Nielsen, 1995), whereas EDC between activated carboxylic acid groups of glutamic or aspartic acid residues and amine groups (Kuijpers et al., 2000). However, how the presence of the covalent cross-bonds induced by these agents affects the physico-chemical structure of the cod gelatin films needs to be studied.

The objective of this study was to investigate the structure of cod gelatin films modified with EDC and TGase by means of the FT-IR spectroscopy and DSC analysis.

2. Materials and methods

2.1. Materials

Fish gelatin was obtained from skins of Baltic cod (*Gadus morhua*) as described by Kołodziejska, Kaczorowski, Piotrowska, and Sadowska (2004). Type A gelatin from pig skins (175 Bloom) was purchased from Sigma Chemical Co. EDC, applied for the chemical modification and commercial TGase, containing 1% of enzyme and 99% of maltodextrin, were purchased from Sigma Chemical Co. and Ajinomoto Co's Transglutaminase Activa®-WM, Japan, respectively. The preparation of TGase was mixed prior to use with cold destilled water in an ice bath for 3 min. Its enzymatic activity, declared by the manufacturer, was 80–130 U/g.

2.2. Chemical and enzymatic modification

Fish-skin gelatin was dissolved in water at room temperature to achieve the final concentration of 5% (w/w). In order to modify cod gelatin film, either EDC or TGase was added to the film-forming solutions, to the final concentration of 30 mM and 0.2 mg/ml, respectively. As we have shown earlier, these concentrations were the most efficient in decreasing the gelatin films' solubility (Piotrowska et al., 2008). The films were formed just after adding of EDC or TGase as described below. The chemical and enzymatic reactions were running during that process.

2.3. Film formation

In all experiments, 20 g of solutions were cast on a rectangle of sides 9.5 cm and 13.5 cm of a polyester surface and spread manually to the outside borders. The films were obtained after water evaporation (24–48 h) at room temperature and at 35–45% relative humidity (RH).

2.4. Attenuated total reflectance Fourier transformation infrared (ATR FT-IR) spectroscopy

The ATR FT-IR spectra of gelatin and gelatin film samples were recorded on a Nicolet 8700 spectrometer (Thermo Electron Scientific Inc., Waltham, MA), using a Golden Gate ATR accessory (Specac) equipped with a single-reflection diamond crystal. The temperature during measurements was kept at 25 ± 0.1 °C using an electronic temperature controller (Specac). For each spectrum, 128 scans were collected with a resolution of 4 cm^{-1}. The spectrometer's EverGlo source was on turbo mode during measurements. The spectrometer and ATR accessory were purged with dry nitrogen to diminish water-vapor contamination of the spectra. All samples were conditioned before their analysis for 7 days in a desiccator containing silica gel.

2.5. Differential scanning calorimetry (DSC)

The samples of films (10 mg) were sealed in aluminum TA pans and then heated at the rate of 10 °C/min in the temperature range of 20–230 °C. A Mettler TA3000 calorimeter (Mettler Instrument, Switzerland), equipped with a TC 10 TA processor and a DSC 30 temperature cell, was used with an empty pan as a reference. The instrument was calibrated with pure indium (T_m = 156.6 °C and ΔH_m = 28.45 J/g). In order to obtain low moisture content material, samples were conditioned before their analysis for 7 days in a desiccator containing silica gel. Analyzes were run in triplicates. The obtained data were processed statistically by performing one-way analysis of variance to determine significant differences among samples, using STATGRAPHICS version 2.1 (Statistical Graphics Corporation, USA). Significance was accepted at $p < 0.05$.

3. Results and discussion

3.1. General

In order to form a film, gelatin was first dissolved in water and then the solution was dried at room temperature. During the both processes, polymer chains underwent conformational transitions which resulted in the development of the network structure. When the films were formed from the gelatin modified by means of EDC or TGase, along with conformational transitions induced by the dissolution and drying processes, also covalent cross-linking induced by the two modifying agents was involved.

3.2. Films characterization by FT-IR spectroscopy

3.2.1. Unmodified cod and pig gelatin films

Table 1 lists the band assignment in the FT-IR spectra of gelatins and resulting gelatin films (Kong & Yu, 2007; Li, Kennedy, Jiang, & Xie, 2006; Muyonga, Cole, & Duodu, 2004).

Fig. 1A shows the changes in the FT-IR spectra of gelatin from cod skins induced by the process of gelatin film formation. For comparison, analogous spectra of gelatin from pig skins are presented in Fig. 1B. An analysis of the patterns of the difference bands made possible to reveal the groups involved in the conformational changes caused by this process.

The first effect which was observed as a result of film formation, was an increased absorption in the region between 3700–3000 cm^{-1}, with the two main peaks appearing at 3450 and 3244 cm^{-1} in the difference spectrum of cod gelatin (Fig. 1A), and the two peaks at 3308 and 3230 cm^{-1} in the difference spectrum of pig gelatin (Fig. 1B). The former pair of peaks was due to the asymmetric and symmetric O–H stretching vibrations (ν_{OH}) of water molecules, and the latter pair due to the N–H stretching vibrations (ν_{NH}) of amides – the first peak, and the symmetric ν_{OH} of water molecules – the second peak. The OH and NH groups led to a shift by 4 cm^{-1} in the maxima of the amide A band of gelatin towards lower wave numbers in the case of the film from cod-skin gelatin, and a shift by 20 cm^{-1} towards higher wave numbers in the case of the film from pig-skin gelatin (Table 1). Sionkowska, Wiśniewski, Skopińska, and Mantovani (2006), examining collagen films, showed that the amide A band, associated with ν_{NH}, was shifted by 14 cm^{-1} to lower wave numbers after solar irradiation.

Table 1
Infrared spectral characteristic of gelatins and resulting gelatin films.

Region	Position in cm^{-1}				Band assignment
	Cod		Pig		
	Gelatin	Gelatin film	Gelatin	Gelatin film	
Amide A	3292	3288	3280	3300	ν_{NH}, ν_{OH}
Amide B	3075	3075	3068	3078	ν_{NH}
	2930	2930	2944	2944	asym ν_{CH_2-}
Amide I	1633	1633	1630	1630	$\nu_{C=O}$, ν_{NH}
Amide II	1524	1538	1523	1541	δ_{NH}, ν_{C-N}, ν_{C-C}
	1448	1451	1444	1450	δ_{CH_2}
	1396	1399	1410	1410	ν^{-}_{COO}
	1334	1334	1334	1334	δ_{CH_2} wagging
Amide III	1232	1238	1235	1235	ν_{C-N}, δ_{NH}
	1080	1080	1080	1080	Skeletal ν_{C-O}
	1030	1030	1032	1032	Skeletal ν_{C-O}

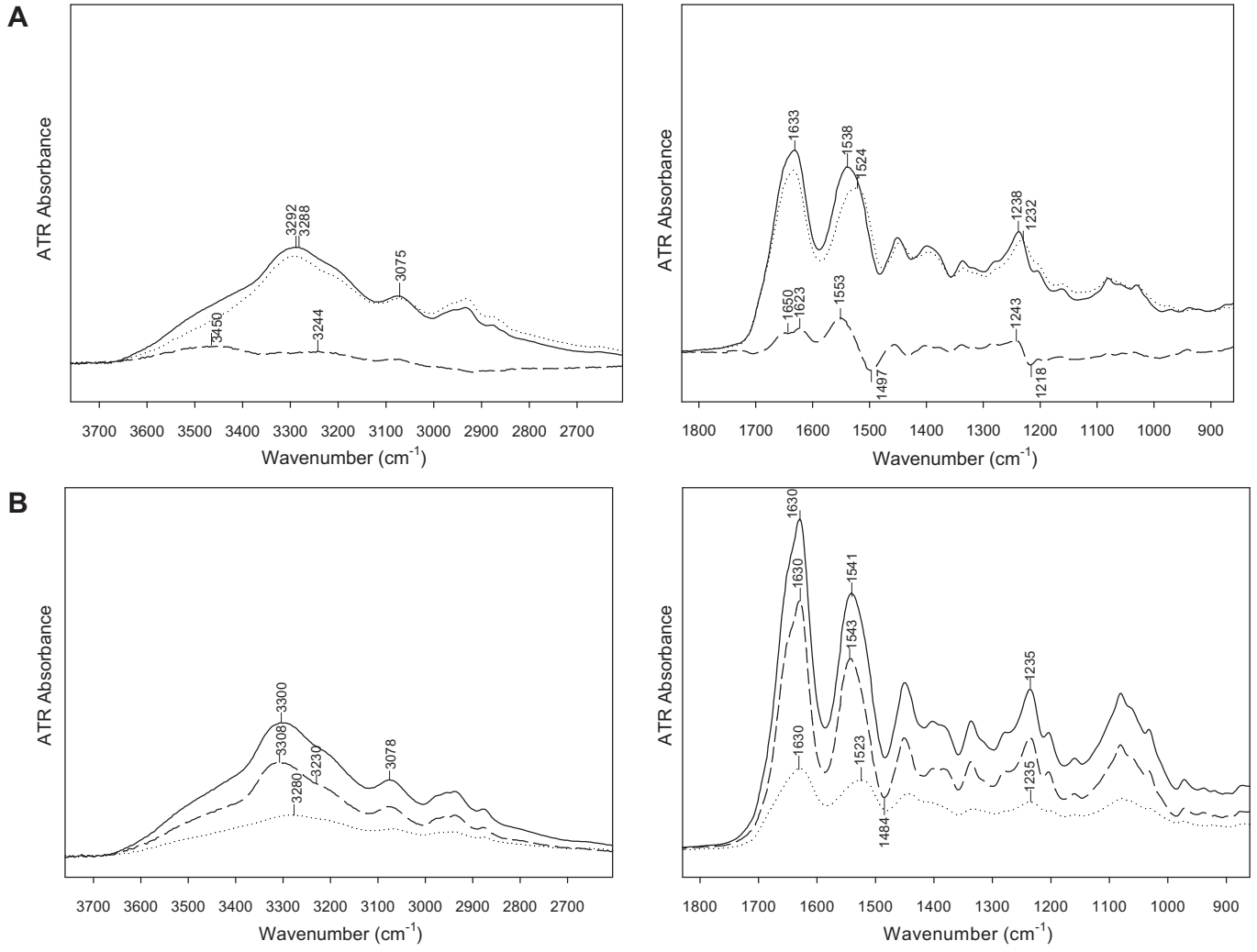

Fig. 1. FT-IR spectra of the gelatin (. . .), gelatin film (—) and difference spectrum of gelatin film from which spectrum of gelatin was subtracted (- - -). Gelatin from cod (A) and pig (B) skins.

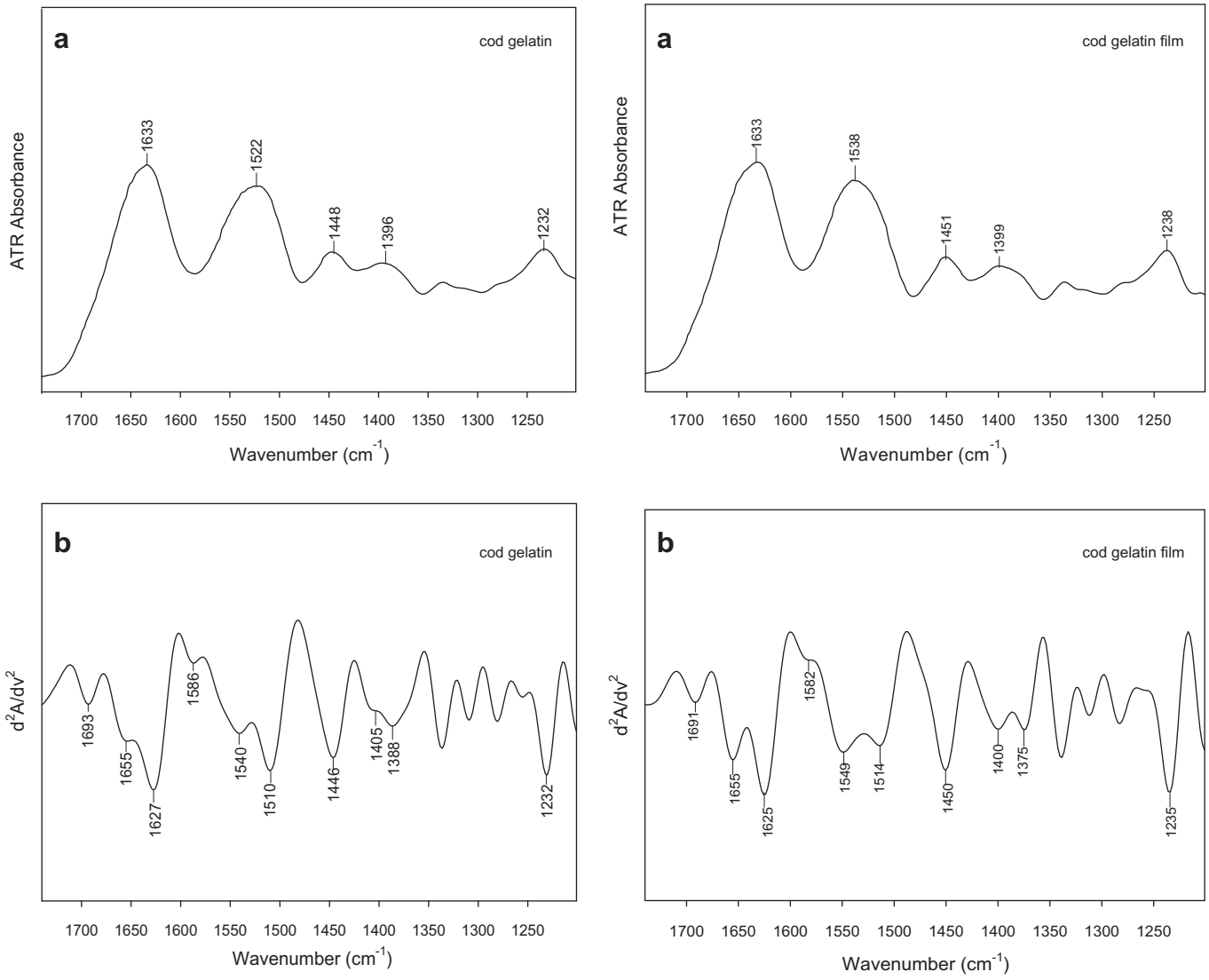

Fig. 2. Absorbance (a) and second-derivative (b) spectra of the gelatin and gelatin film from cod skins.

This shift was interpreted by the authors to result from the scission of hydrogen bonds necessary to maintain the helical structure of collagen due to exposure to UV irradiation. Hence, the shift of the amide A band of pig gelatin by 20 cm^{-1} towards higher wave numbers after the film formation may be interpreted as a result of the formation of the hydrogen bonds in the recovering helical structure. The appearance in the differential spectrum of the peak at 3308 cm^{-1}, assigned to $\nu_{NH,}$ could confirm the formation of these bonds. In turn, the slight shift of the amide A band of cod gelatin by 4 cm^{-1} towards lower wave numbers after the film formation, and the appearance in the differential spectrum of the peaks assigned to $\nu_{OH,}$ may indicate the involvement of the OH groups in hydrogen bonding in the obtained film. Typically, a shift towards lower wave numbers of the 3700–3000 cm^{-1} band has been described as indicative of water-mediated hydrogen bonding (Silva et al., 2008; Yakimets et al., 2007). Thus, the observed feature could point at a water-to-amide hydrogen bonds in the case of the film from cod-skin gelatin, and amide-to-amide rather than water-to-amide hydrogen bonds in the case of the film from pig-skin gelatin. Subsequently, an increased intensity in the difference band at 1623 cm^{-1} with a shoulder at 1650 cm^{-1} in the spectrum of cod gelatin, and a much larger increase at 1630 cm^{-1} in the spectrum of pig gelatin, contributed, due to the solvent water, to the enhancement of the amide I band in the spectra of both gelatin films. The water molecules were, most likely, hydrogen-bonded to carbonyl groups of gelatin backbone (Payne & Veis, 1988). However, the higher intensity of the differential peak in the pig gelatin spectrum may result from a greater contribution of H–O–H bending vibrations in water molecules in that case. In the amide II band region, an increase of absorbance at 1553 cm^{-1} and a decrease at 1497 cm^{-1} in the difference band of cod, and an increase at 1543 cm^{-1} and a decrease at 1484 cm^{-1} in the difference band of pig gelatin, were associated with a shift towards higher wave numbers in the maximum of the amide II band in the spectra of both gelatin films (Table 1). Yakimets et al. (2005) noted a similar effect by examining the hydrated films from bovine gelatin. Because of the nature of the amide II band, combined ν_{CN} δ_{NH}, which is very much influenced by hydration, the authors interpreted this effect as indicating increased level of protein backbone hydration. The authors also used the above argument to explain the changes observed in a difference band of gelatin film in the amide III band region, where, like in our studies in the case of the film from cod gelatin, increasing hydration caused a frequency shift toward higher wave numbers (Table 1).

By comparing the spectra of both gelatins one could see that pig gelatin entrapped considerably more water to form a film than did

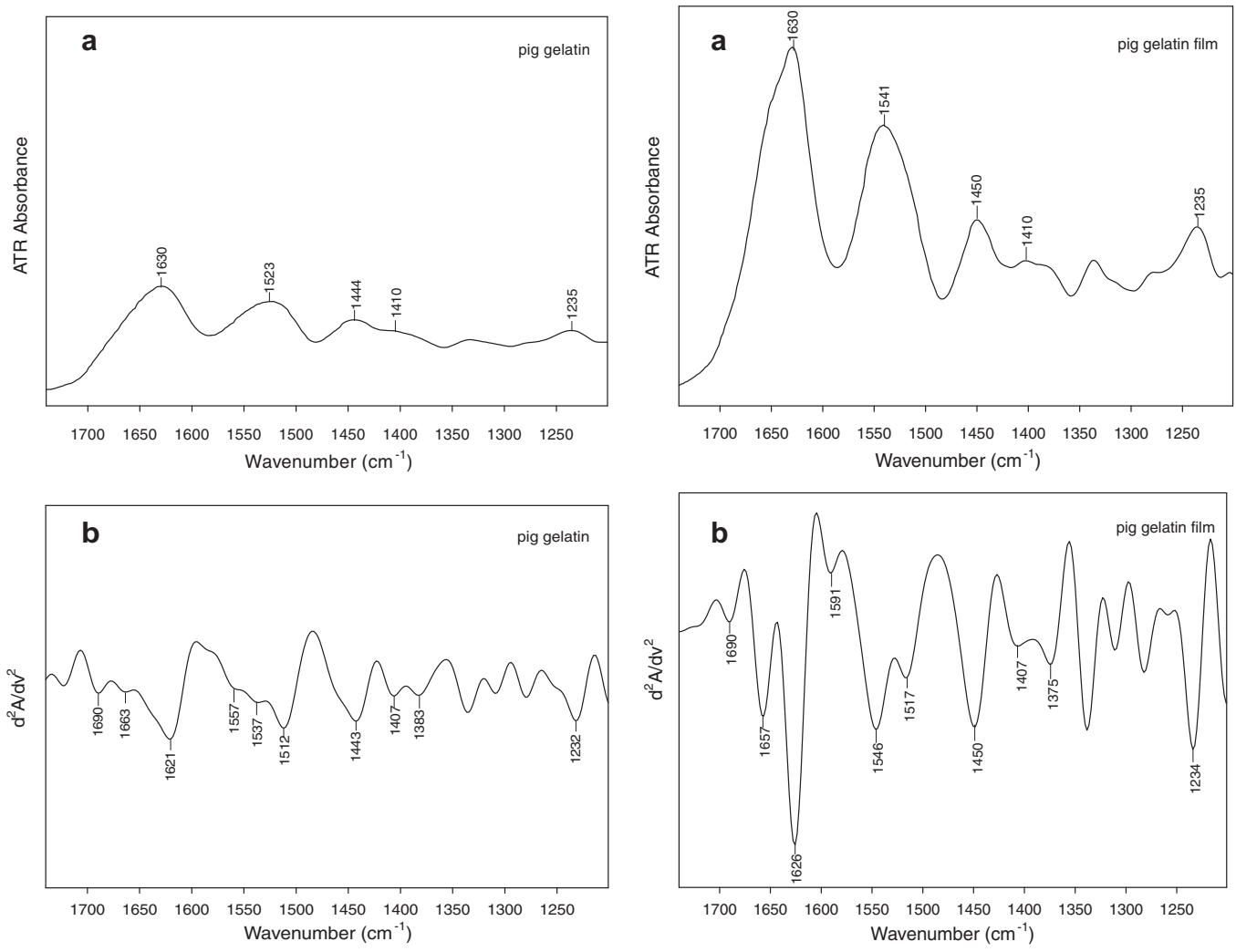

Fig. 3. Absorbance (a) and second-derivative (b) spectra of the gelatin and gelatin film from pig skins.

cod gelatin. In the case of gelatin from cod-skins, the gelatin chains interacted with solvent water, resulting in the hydration of chains. In the case of gelatin from pig-skins, the water molecules formed probably extra hydrous layers which surrounded hydrated as well as hydrogen-bonded unhydrated chains. Thus, the structure of pig gelatin film contained a higher number of hydrogen bonds, involving water-to-amide, amide-to-amide and water-to-water hydrogen bonds, than the structure of cod gelatin film involving first of all water-to-amide hydrogen bonds. Therefore, it could be supposed that the structure of film from pig gelatin was characterized by a higher degree of molecular order than the structure of cod gelatin film.

The changes in the structure of proteins arising as a result of solvent water binding can be investigated by applying the second-derivative procedure (Byler & Susi, 1988; Haris & Severcan, 1999; Kong & Yu, 2007). Hence, this procedure was employed here to study a number of overlapping peaks, resolved in their components. In the amide I band region, the most informative band on the secondary structure of protein, the second-derivative spectra of both gelatins exhibited three peaks (Fig. 2 and 3). The component peak of the smallest intensity at about 1690 cm^{-1} was attributed to intermolecular associations, and that of the largest intensity at about 1620 cm^{-1} was due to imide residues (and partly to β-sheet) (Muyonga et al., 2004; Taravel & Domard, 1995). According to Nevskaya, Chirgadze, and Yu (1976), for the α-helical proteins the observed frequency of the amide I band lies near 1663 cm^{-1}, and of the amide II band near 1537 cm^{-1}. In our present second-derivative spectra, the weakly developed peaks at 1655 or 1663 cm^{-1} in the amide I band and at 1540 or 1537 cm^{-1} in the amide II band, in the spectrum of cod or pig gelatin, respectively, were observed.

The second-derivative spectra of both gelatin films displayed all these peaks. Furthermore, the peaks which were attributed to helical structure in gelatin, were much more developed in the film, particularly in the case of the film from pig gelatin. Comparison of the intensity of the peaks at about 1663 cm^{-1} in the amide I band and at about 1537 cm^{-1} in the amide II band clearly pointed that the helical structure is much better recovered in the case of the film from pig than from cod gelatin. The intensity of the peak at 1626 cm^{-1}, attributed to imide residues (Muyonga et al., 2004; Taravel & Domard, 1995), implied that in the stabilization of the latter structure in the pig gelatin film an active role was played by imino acids.

3.2.2. Fish gelatin film modified with EDC and TGase

The cod gelatin films modified with EDC or TGase are spectrally characterized in Table 2. Fig. 4 shows the changes in the spectrum of cod gelatin film caused by chemical (Fig. 4A) or enzymatic (Fig. 4B) modification, using EDC or TGase, respectively. Generally, an increase in the absorbance in the amide A, amide I, amide II and

Table 2
Infrared spectral characteristic of cod gelatin films modified with either EDC or TG.

Region	Position in cm^{-1}	
	Film modified with EDC	Film modified with TGase
Amide A	3280	3278
Amide B	3068	3075
	2930	2930
Amide I	1633	1633
Amide II	1532	1533
	1448	1448
	1404	1406
	1334	1332
Amide III	1240	1241
	1080	1080
	1030	1023

amide III band region in the spectra of gelatin films modified with both agents was observed. In detail, the increased absorption in the amide A band region, with two peaks at 3370 and 3258 cm^{-1} in the difference spectra, due to the bounded ν_{NH} of primary amides – the first peak, and the symmetric ν_{OH} of water molecules – the second peak, was combined with a shift of the maximum of that band towards lower wave numbers (Table 1 and 2). The formation of the covalent linkages, between amine groups and either γ-carboxyamide groups of peptide-bound glutamine residues in the case of using TGase or activated carboxylic acid groups of glutamic or aspartic acid residues in the case of using EDC, results in the formation iso-peptide bonds (Kuijpers et al., 2000). Thus, as the cross-linking progresses, the number of bounded NH groups increases and this is reflected by the first peak in the difference spectrum. The presence of the second peak and the shift of the amide I band towards lower wave numbers, indicate water-mediated hydrogen bonding. Next, an increase of absorbance in the difference bands at about 1633 cm^{-1} and 1523 cm^{-1} contributed to an enhancement of the amide I and the amide II bands, respectively. The cross-linking of gelatin film favored probably the changes in the protein structure which improved its hydration ability. However, the intensity of the differential peak in the amide II band region was lower than the peak in the amide I band region. These data agree with earlier studies on collagen cross-linking with TGase (Garcia, Collighan, Griffin, & Pandit, 2007) and EDC (Wang et al., 2003). These studies showed that as a result of the formation of covalent bonds between the γ-carbonyl group of glutamine residue and the ε-amino group of a lysine residue, the intensity of the

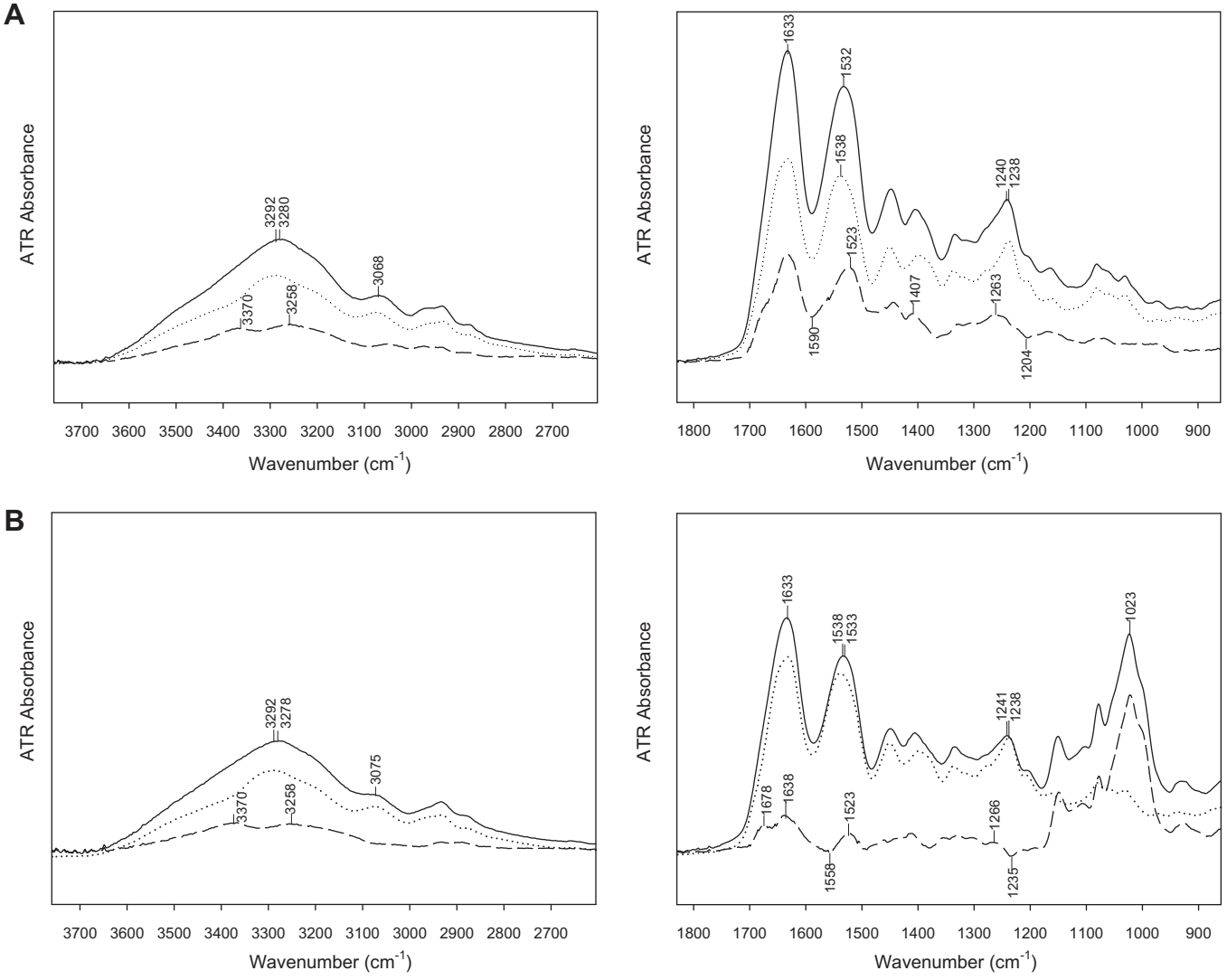

Fig. 4. FT-IR spectra of the cod gelatin film (. . ..), modified cod gelatin film (—) and difference spectrum of modified cod gelatin film from which spectrum of cod gelatin film was subtracted (- - -). Cod gelatin film modified with EDC (A) and TGase (B).

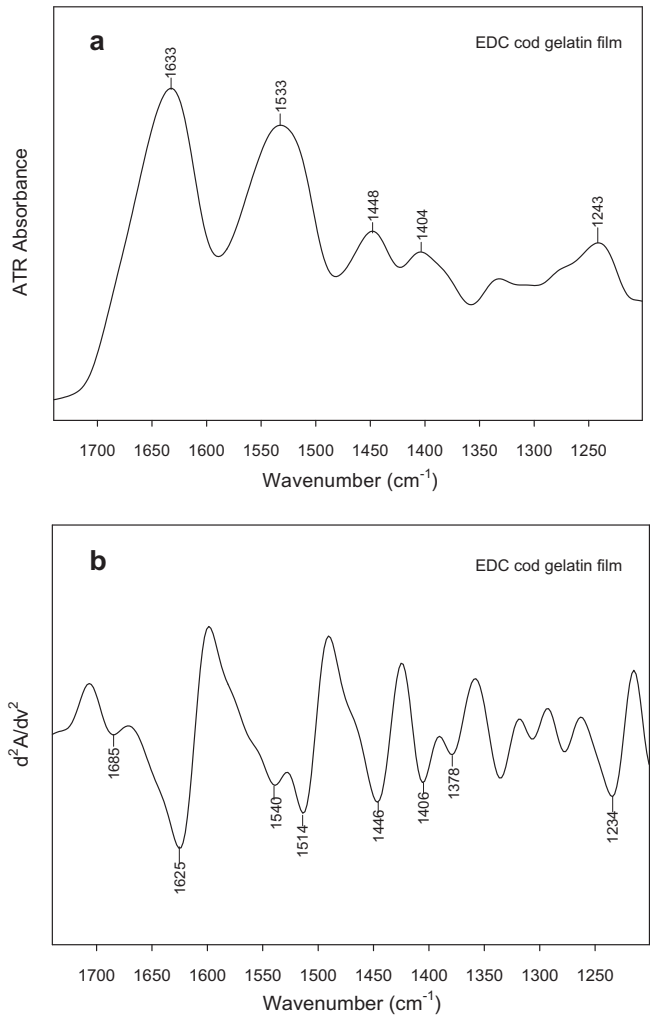

Fig. 5. Absorbance (a) and second-derivative (b) spectra of the cod gelatin film modified with EDC.

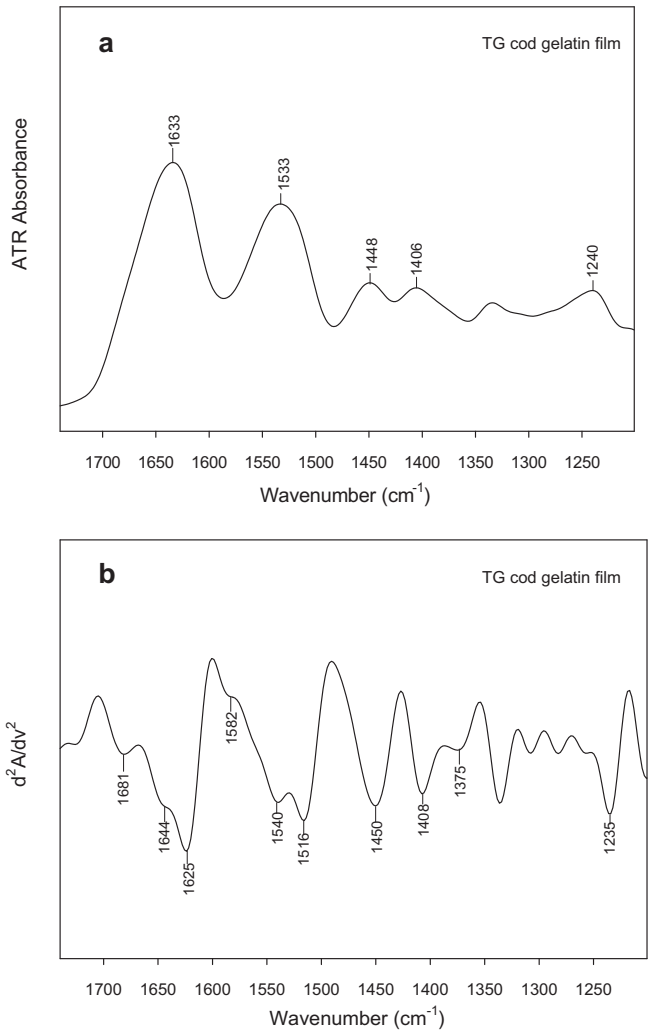

Fig. 6. Absorbance (a) and second-derivative (b) spectra of the cod gelatin film modified with TGase.

amide I band increased because the strength of C═O stretching ($\nu_{C=O}$) and N–H bending (δ_{NH}) vibrations in the new covalent bonds was increased. Simultaneously, the intensity of the amide II band decreased because the $-NH_2$ free groups in the collagen molecules were changed into N–H groups after their cross-linking, and the intensity of $-NH_2$ band in the collagen molecules is stronger than that of NH (Wang et al., 2003). In the present study, in the spectra of films from cross-linked gelatin, the bending vibrations of N–H bonds (δ_{NH}) in the amide II band region moved additionally to lower wave numbers in comparison to the spectrum of the film from unmodified gelatin (Table 1 and 2). It was likely that the energy of intra- molecular interaction involving these groups decreased, because the formation of the film from cross-linked gelatin required conformational changes of gelatin to ensure the film formation. In the difference spectrum of the gelatin cross-linked with EDC, besides the above-discussed changes, one could also observe a clear decrease in the intensity of the band near 1400 cm^{-1} when compared with the film obtained from unmodified gelatin. This evidently indicated that the carbonyl groups took part in the cross-linking process. The spectrum of the film from gelatin modified with TGase exhibited an additional band with the maximum at 1023 cm^{-1}. On the basis of the TGase spectrum (spectrum not shown) it was determined that this band came from the saccharide structure of maltodextrin incorporated into the preparation of commercial TGase.

All these spectral changes indicated the formation of the structure characterized by the presence of a large number of inter-chain cross-linkages. Furthermore, the main difference in the cross-linking induced by both agents lies in the fact that in the case of using of TGase – glutamine residues participate in the cross-linking, and in the case of using of EDC – carboxylic acid groups of glutamic or aspartic acid residues are involved.

More detailed information on the created structure was obtained from the second-derivative analysis. Fig. 5 and 6 present second-derivative spectra of the chemical (Fig. 5) and enzymatic modified (Fig. 6) gelatin film. There were either two or three component peaks in the amide I band region in the spectrum of chemical or enzymatic modified gelatin film, respectively, attributed, in turn, to intermolecular associations at about 1680 cm^{-1}, to imide residues at 1625 cm^{-1}, and to unordered structure at 1644 cm^{-1}. In the amide II band region two weakly developed peaks at 1540 and about 1515 cm^{-1} could be recognized in both spectra. Comparison of the second-derivative spectra of modified and unmodified gelatin films from cod-skins (Fig. 2, 5 and 6), and also that of unmodified gelatin from pig-skins (Fig. 3), shows that gelatin macromolecules, after their cross-linking and film forming, took the conformation with no indications of helical ordering. Thus, it could suggest that the large number of inter-chain cross-linkages which replaced the water-to-amide interactions make ordered structure difficult to recover for gelatin.

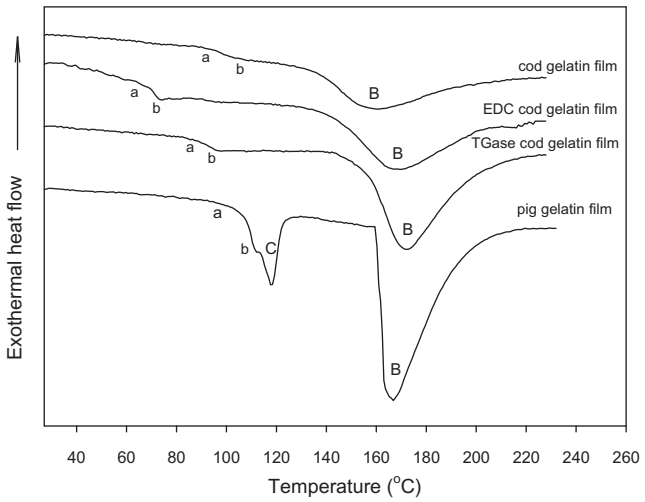

Fig. 7. DSC thermograms of unmodified and modified with EDC and TGase cod gelatin film. Thermogram of pig gelatin film is shown for comparison.

3.3. Films characterization by DSC analysis

The DSC thermograms of gelatin films, shown in Fig. 7, displayed two transitions due to heating. The temperature of the first transition was determined as a point of a shift in the thermogram line (line ab), whereas the temperature of the second one was evaluated as the maximum of its endothermic peak (peak B). These temperatures represent the glass (T_g) and the melting (T_m) transition, respectively. For gelatin, T_m is referred to as the helix–coil transition (Sionkowska, 2000; Sobral & Habitane, 2001). This phenomenon is accompanied by a release of water molecules which form hydrogen bonded bridges stabilizing the helix. Thus, the endothermic peak with a maximum at T_m arises from water molecules, which are released during the helix–coil transition.

The results presented in Fig. 7 are in agreement with former findings, and are considered as a classical behaviour of gelatin (Sobral & Habitane, 2001). However, the thermogram of the film from pig gelatin revealed one peak more, which was visible between the glass and the melting transition. Sobral and Habitane (2001), who observed a similar phenomenon in the case of pig-skin gelatin supposed that this peak must be due to a helix–coil transition of some fraction of the gelatin. In a review paper on food product stability, Schenz (1995), following Levine and Slade (1990), indicated that, as a sample of some food polymer system is heated it progresses from the glassy state into a rubbery region which falls between T_g and T_m. Recently, Rahman et al. (2008), who investigated the thermal properties of gelatin extracted from tuna skin and commercial mammalian gelatin using the modulated differential scanning calorimetry, argued that the peaks observed after the glass transition and before the melting on the thermograms of mammalian gelatin may also result from the transformation of glassy to rubbery state.

Table 3 shows that the T_g of film from cod gelatin was lower by 10 °C than that from pig gelatin and decreased even more for films from cross-linked cod gelatin. Rahman et al. (2008) showed that the gelatin from tuna also demonstrated a lower T_g than those of bovine and pig gelatin, and this difference was probably connected with a larger moisture content that fish gelatin can adsorb in comparison with a mammalian source gelatin. The decrease of T_g by 28 °C on EDC and by 7 °C on TGase cross-linking of cod gelatin demonstrated the well-known plasticizing effect of water. Cristiano et al. (2010) showed that the T_g of film from bovine-skin gelatin decreased from 215 to 145 °C after its cross-linking by means of EDC. According to these authors, a very high cross-linking degree of

Table 3
DSC calorimetric parameters of gelatin films: temperatures of glass (T_g) and melting (T_m) transition and melting enthalpy (AH).

Film	T_g (°C)	T_m (°C)	AH (J/g)
Cod gelatin	99[a]	161[a]	62.8[a]
Modified with EDC	71[b]	169[b]	72.5[b]
Modified with TGase	92[a]	172[b]	98.4[c]
Pig gelatin	109[c], 118	166[b]	152.7[d]

Values in the columns marked with various letters are significantly different at $p < 0.05$.

that film have indicated that the treatment with EDC favored the exposure of the free hydroxyl groups. Therefore, the decrease in T_g was interpreted by the authors as the effect of plasticization of film cross-linked with EDC induced by the water molecules. The same effect was also observed in a soy protein isolate cross-linked with TGase (Tang, Cheng, Li, & Yang, 2006). The formation of a new covalent bonds in that case produced changes in the protein structure, which improved its hydration ability. Moreover, it is possible that the T_g of the modified films was decreased due to the different alignment of gelatin molecules in which the order or compact structure after modification was decreased, which resulted in the increased free-volume in the film matrix.

The results presented in Table 3 show that the T_m of the film from pig gelatin was higher by 5 °C than that from cod gelatin. Generally, mammalian gelatin with a higher amount of imino acids content has a higher melting temperature than fish gelatin containing a lower amount of imino acids (Gómez-Guillén et al., 2002; te Nijenhuis, 1997). However, Rahman et al. (2008) reported that the T_m of pig and tuna gelatin films conditioned at 53% RH had the values of 156 and 204 °C, respectively. For the film from pig gelatin, this T_m was different from that observed by Silva et al. (2008), which was equal to 89 °C when the film was conditioned at 55–65% RH. A similar result (T_m = 89 °C) was reported by Chiou et al. (2008), however, the authors did not provide the value of RH at which their films were conditioned. In the case of the fish gelatin, for a film obtained from commercial fish gelatin conditioned at 50% RH, also a value of T_m = 125 °C was reported by Yi, Kim, Bae, Whiteside, and Park (2006). The differences in T_m which are reported in the literature are due to different profiles of gelatin sources and different moisture content or water activity.

Furthermore, the thermogram of the film from pig gelatin, unlike that from cod gelatin, indicated a more ordered structure showed by a narrower melting peak. The much better recovered helical structure in the film from pig than from cod gelatin, which was stabilized not only by intra-molecular hydrogen bonds but also by the water layer, may explain its significantly higher melting enthalpy (ΔH) value (Table 3). The cross-linking of cod gelatin was reflected by an increase in T_m by 7 °C for EDC and by 10 °C for TGase modified gelatin films. The increase in thermal stability of the films from cold-water fish gelatin cross-linked with TGase (Yi et al., 2006), and also form pig gelatin cross-linked with glutaraldehyde (Bigi et al., 2000; Bigi et al., 2001) has been related to the formation of covalent cross-links. However, the increase in T_m is usually reflected by a decrease in ΔH values. Such a feature was observed by Bigi et al. (2001) and Bigi, Panzavolta, and Rubini (2004) who investigated chemical cross-linked pig gelatin, and by de de Carvalho and Grosso (2004) who studied chemical cross-linked bovine gelatin. According to these authors, this is due to the presence of covalent bonds which break exothermically, unlike hydrogen bonds which break endothermically. In our studies, the ΔH values of films from cod gelatin cross-linked with EDC or TGase increased, which could suggest that a larger number of hydrogen than covalent bonds were formed in cross-linked films.

However, more detailed studies are necessary to confirm this explanation.

4. Conclusions

A higher number of hydrogen bonds formed in the structure of the film from pig than from cod gelatin results in a much better recovering of helical structure in the case of the pig gelatin film. This helical structure, in turn, is the reason for significantly higher value of melting enthalpy of this film than that from cod gelatin. After modification of cod gelatin with EDC or TGase, the inter-chain cross-linkages formed in the films make ordered structure difficult to recover for gelatin. However, the formation of covalent cross-links on EDC and TGase, contributes to an increase of melting temperature by 7 °C and 10 °C, respectively, and to a decrease of glass temperature by 28 °C and 7 °C on EDC and TGase cross-linking, respectively. The latter demonstrates the plasticizing effect of water due to changes in the protein structure caused by cross-linking.

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