Consequences of UV Irradiation on the Zirconium Tanned Collagen: A Molecular Level Study

by
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Abstract

UV and heat resistance are very important qualities for many leather applications especially, automobile upholstery. This study explores the effect of UV irradiation on zirconium tanned collagen. The changes in the physiochemical characteristics of native collagen upon UV irradi ation differ from that of the zirconium tanned collagen. The UV absorption characteristics of crosslinked collagen increases, which is attributed to the formation of photoproducts on UV radiation. Zirconium crosslinking has been found to reduce this formation when the duration of irradiation is less. There is a gradual decrease in the emission maxima of crosslinked collagen on increasing the time of irradiation, which is ascribed to conformational changes of the aromatic amino acids residue (tyrosine). The duration of irradiation plays a critical role in the conformational changes brought about by it. Thus, the crosslinking brought about by zirconium aids in imparting stability to collagen against UV irradiation.

Resumen

UV y resistencia al calor son cualidades muy importantes para muchas aplicaciones de cuero especialmente, la tapicería de automóviles. Este estudio explora el efecto de la irradiación ultravioleta en colágenos curtidos al circonio. Los cambios en las características físico-químicas de colágeno nativo a la irradiación UV difieren del colágeno curtido al circonio. Las características de absorción UV del colágeno reticulado aumenta, lo que se atribuye a la formación de foto-productos por la irrradiación UV. Se ha encontrado la reticulación con circonio para reducir esta formación cuando la duración de la irradiación es menor. Hay una disminución gradual en la máxima emisión de colágeno reticulado aumentando el tiempo de irradiación, que se atribuye a cambios de configuración de los residuales de aminoácidos aromáticos (tirosina). La duración de la irradiación desempeña un papel fundamental en los cambios producidos por configuración ella. De este modo, la reticulación provocada por circonio ayuda a impartir estabilidad al colágeno en contra de la irradiación ultravioleta.

Introduction

Collagen is the major protein present in skin and is also the leather making protein. As such, collagen is a material, with good biological compatibility that can be degraded into physiologically tolerable compounds.[1–3] Ultraviolet (UV) radiations are known to cause harmful effects on human beings. Research has been carried out on the effect of UV radiation on collagen over a long period of time.[4-8] Studies on the effect of UV radiation on collagen have a wider application in the medical, biomaterial and tanning industries.

Temperature, UV radiation, and humidity are key environmental factors that affect leather properties. UV and heat can have a detrimental effect on the durability of automotive leather, especially for instrument panels and consoles, where very high temperatures can be reached. UV and heat resistance are very important qualities for automobile applications. UV and heat are known to be more detrimental to chrome-free leather than chrome-tanned leather, especially when it concerns the colourfastness and mechanical properties. The role of humidity and its interaction with UV radiation and temperature on leather properties, however, are not clear to the leather industry. Hence, in the present study an attempt has been made to understand the effect of UV radiation on zirconium tanned collagen at the molecular level.

Metal ions find predominant application in crosslinking of collagen for various end uses as those in the tanning industry and also biomedical application. Presently, research on replacement for chromium as a tanning salt is actively pursued for various environmental reasons. Zirconium, though known for long for its tanning potential, has never been successful commercially for reasons like high acidity, drawn grain, lack of fullness etc. However, it has been shown that zirconium oxychloride can be employed as a

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tanning agent for manufacture of variety of leathers.[9,10] Organozir, a zirconium tanning salt complexed with organic moieties has also been shown to have better tanning capabilities.[11] Zirconium tanning produces white leathers, which meets fashion requirements for pastel shades and also it is used for making speciality leathers. The physical and optical properties of zirconium tanned collagen affected by UV irradiation have been detailed in this study. The main objective of study is to investigate the molecular level changes that takes place in the zirconium tanned collagen under UV irradiation and to compare the structural changes of the UV radiated native collagen and the collagen tanned with zirconium.

Materials and Methods

Collagen Solution

6-month old male albino rats (Wistar strain) were sacrificed and tails were obtained from them and stored at –20ºC. The tendons were teased from the tails after thawing them. Acid soluble rat-tail tendon (RTT) type I collagen was isolated according to the method described by Chandrakasan, et al and the concentration was estimated by hydroxy proline estimation as per Woessner method.[12,13] Collagen was extracted and purified from rat-tail tendon. All the procedures described below were carried out at temperature around at 4ºC. Tendons teased were washed in 0.9% solution of saline water. The tendons were stirred overnight at 0.5 M acetic acid. Protease inhibitors like N-ethylmaleimide (1 mM) & Phenyl sulfonyl fluoride (PMSF) (1 mM) were added to the mixture. The solution was then filtered and centrifuged at 50,000 RPM for 40 minutes and the residue was discarded. The supernatant containing collagen solution was precipitated with slow addition of 5% (w/v) sodium chloride under stirring for 30 minutes and left overnight. The residue was redissolved in 0.5 M acetic acid by stirring them over night. The re-dissolved solution was centrifuged at 50,000 RPM for 40 minutes and the supernatant collected was dialyzed extensively against 0.02 M Di-sodium hydrogen phosphate buffer with 2 to 3 changes of buffer each day until the thick precipitate was obtained. The precipitated collagen solution was centrifuged at 35,000 RPM for 30 minutes and the precipitate was re-dissolved in 0.5 M acetic acid. The dissolved solution was centrifuged at 50,000 RPM for 40 minutes and the supernatant was dialyzed against 0.05M acetic acid buffer for 2 days. After dialysis the solution was centrifuged at 50,000 RPM for 40 minutes and purified collagen was stored at 4ºC. Collagen in solution (0.05 M acetic acid) is dialyzed further using a 12,000 molecular weight cut off dialysis tubing against 5 mM acetic acid overnight at 4ºC, with two changes of dialysate, to remove small peptide degradation products. The solution is then centrifuged at 100,000 RPM for one hour in a centrifuge to remove large aggregates. The supernatant from this centrifugation containing soluble collagen solution is used for studies.

Stabilization of Collagen using Zirconium and UV Irradiation

A known concentration of collagen solution (0.6 μM) was treated with zirconium oxychloride in the molar ratio of collagen to zirconium as 1:100, and was then incubated at room temperature (25ºC) for overnight. Solutions were irradiated under air at room temperature using a quantum yield photoreactor (model 2001, Applied photophysics Ltd., London), with 250 W medium pressure mercury lamp, which emits light mainly at a wavelength of 330 nm. Irradiation experiments were carried out in a quartz cuvette at a distance of 20 cm from the light source for various time intervals. All measurements were performed in the same conditions of temperature and humidity to avoid any influence on the physiochemical properties of collagen.

Electronic Spectral Studies and Fluorescence Studies

The UV absorption spectra for native and zirconium tanned collagen solution before and after irradiation were recorded using Perkin-Elmer Lambda 35 spectrophotometer. The emission spectra for native and zirconium treated collagen solution before and after irradiation were recorded using Cary eclipse fluorescence spectrophotometer from Varian. The solutions were excited with light of wavelength 270 nm and the emission at 290 nm was monitored. The concentration of collagen used was 0.6 µM. The ratio of collagen: zirconium was maintained as 1:100.

Circular Dichroism Studies

Circular Dichroism (CD) spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right and left circularly polarized light (rather than the commonly used absorbance of isotropic light) by a substance. It has been shown that CD spectra between 260 and approximately 180 nm can be analyzed for the different secondary structural types: alpha helix, parallel and antiparallel beta sheet, turn, and other. Modern secondary structure determination by CD are reported to achieve accuracies of 0.97 for helices, 0.75 for beta sheet, 0.50 for turns, and 0.89 for other structure types.

CD spectra were measured using Jasco 715 Circular Dichroism spectropolarimeter using a quartz cell with a light path of 1 mm at 25ºC, with 3 scans for each sample. CD spectra were recorded in the far UV region (190-250 nm), under nitrogen, to estimate the conformational changes of native and zirconium treated collagen samples before and after irradiation. To study the effect of duration of irradiation on the conformation of collagen, aqueous solution of collagen (0.6×10^{-6} M) was incubated with zirconium in the ratio of 1:100 for 18 h at 25ºC and irradiated for 15 min, 30 min, 1 h and 2 h.

RESULTS AND DISCUSSION

Collagen on irradiation with UV is known to undergo chemical and physical changes.[14] A lot of interest has been developed to study this effect of UV radiation on physical properties of crosslinked collagen. Recently, it has been reported that aldehyde, vegetable and chromium crosslinking imparts better stability against UV irradiation.[15-17] In this study, the effect of UV radiation on zirconium tanned collagen has been investigated. The crosslinking of collagen with zirconium has been done using zirconium oxychloride. The concentration of the collagen was fixed to 0.6 µM and it has been tanned with zirconium in the ratio 1:100 (collagen: zirconium).

Electronic Spectral Studies

The electronic absorption spectra for collagen and collagen in presence of zirconium before and after irradiation are given in Figures 1 and 2, respectively. From the Fig. 1, the intensity of the peak is found to increase on increasing the time of irradiation. This is attributed to the increase in photoproducts formed due to irradiation of the aromatic amino acids, tyrosine and phenylalanine. During radiolysis of tyrosine, dityrosine formation could occur. It can be seen from Fig. 2 that the response of zirconium tanned collagen is different from that of native collagen. The initial time of irradiation (up to 30 min) is found to decrease the absorbance, which has been followed by an increase in the absorbance as the time of irradiation increases. Thus, the crosslinking brought about by metal ion zirconium aids in imparting the stability to collagen to a certain extent and it is known that zirconium has crosslinking ability with collagen.[18] The studies on chrome and vegetable tanned collagen has also shown similar trend.

Circular Dichroic Studies

The changes in physiochemical properties can be due to changes in the conformation of the protein; hence, CD studies were carried out. In the far UV region, collagen exhibits a minimum at 197 nm and a maximum at 220 nm with a cross over point at about 210 nm. The maximum at 220 nm in CD spectrum of native collagen solution is characteristic of triple folded helix.[19] The CD spectrum of native collagen obtained in this study is similar to those reported in the literature.[20,21] The CD spectra of native collagen which is non-radiated, UV- radiated for 15 min, 30 min, 1 h and 2 h are shown in Fig. 3. It could be seen from the figure that there is a constant increase in the molar ellipticity of the negative peak with the time of irradiation. Also, the molar ellipticity value for non radiated collagen is lesser when compared to that of the radiated samples. The value increases as the time of irradiation increases till 30 min, followed by a small gradual decrease in 1 hour radiated collagen. There is also greater change in helicity observed after 2 h of irradiation. This shows that prolonged irradiation has a pronounced effect on the secondary structure of collagen. The CD spectra of collagen in the presence of zirconium which is non-radiated, UV- radiated for 15 min, 30 min, 1 h and 2 h are shown in Fig. 4. It is seen from the figure that the way zirconium tanned collagen responds to UV radiation is in different pattern when compared to the native collagen. It is seen that the molar ellipticity has been decreased after 15 min and 30 min irradiation under UV, which is then followed by an increase in the molar ellipticity values. Also there are more changes in the molar ellipticity after 1 and 2 hr of irradiation. In the case of aldehydes tanned collagen it was observed that there is denaturation after 2 hr of irradiation.[15] These results can be correlated with electronic spectral studies results where it was seen that zirconium imparts stability for lesser time of irradiation and on prolonged irradiation the stability imparted decreases.

Evaluation using RPN Values

The parameter Rpn denotes the ratio of positive peak intensity over negative peak intensity. It is a characteristic ratio for the triple helical conformation of collagen and collagen like peptide.[22,23] The Rpn values for native collagen and collagen treated with zirconium, before and after UV radiation are given in Table 1. From the table, it is apparent that the there is a continuous decrease in the Rpn ratio of the native collagen. The Rpn value for zirconium tanned collagen increases after 15 and 30 min irradiation followed by a decrease for 1and 2 h irradiation. This also shows that stability imparted by zirconium depends on duration of irradiation.

TABLE I
RPN values of native collagen solution (0.6 µM) and zirconium tanned collagen, before and after irradiation

Time of irradiation (min)	Rpn ratio (characteristic ratio)	
	collagen	zirconium
0	0.153 ± 0.002	0.144 ± 0.003
15	0.152 ± 0.001	0.148 ± 0.001
30	0.151 ± 0.002	0.146 ± 0.001
60	0.144 ± 0.001	0.139 ± 0.002
120	0.121 ± 0.001	0.123 ± 0.001

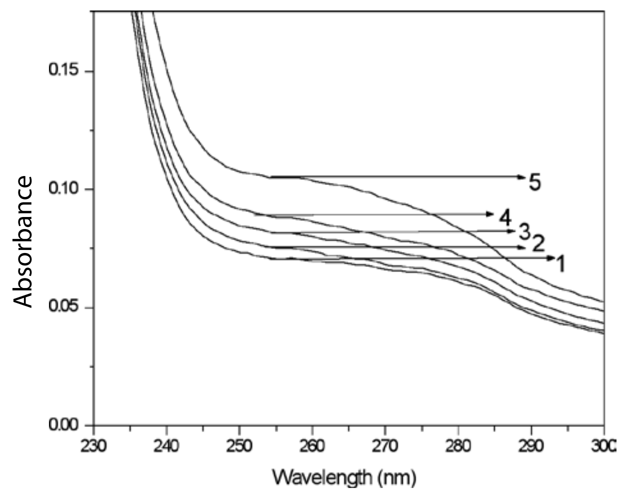

Figure 1: UV absorption spectra for native collagen before and after UV radiation.(1- Non Radiated, 2- 15 min, 3-30 min, 4-1 hour, 5- 2hour Irradiation)

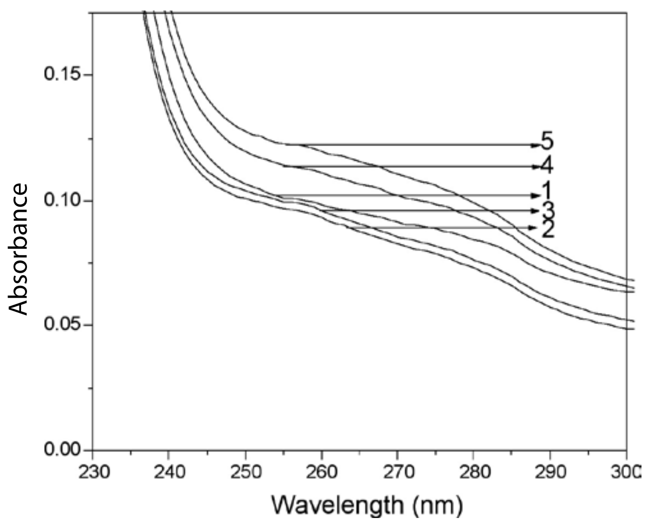

Figure 2: UV absorption spectra for collagen tanned with zirconium before and after UV radiation.(1- Non radiated, 2- 15 min, 3-30 min, 4-1 hour, 5-2h Irradiation)

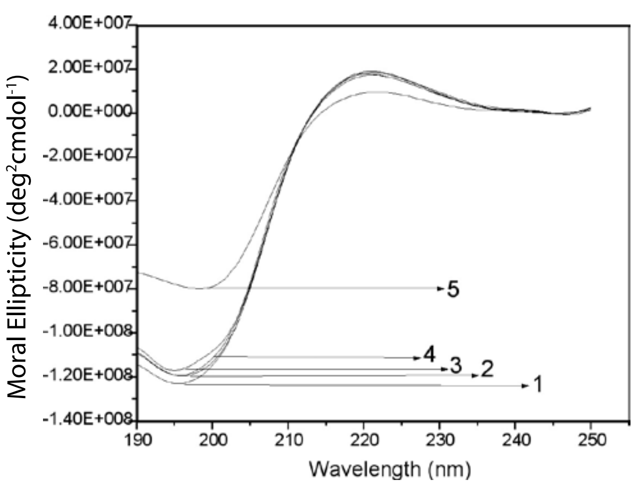

Figure 3: CD spectra of native collagen (0.6 μM) irradiated for various time intervals. (1- 0 min; 2-15 min; 3-30 min; 4-1 h; 5- 2 h irradiation)

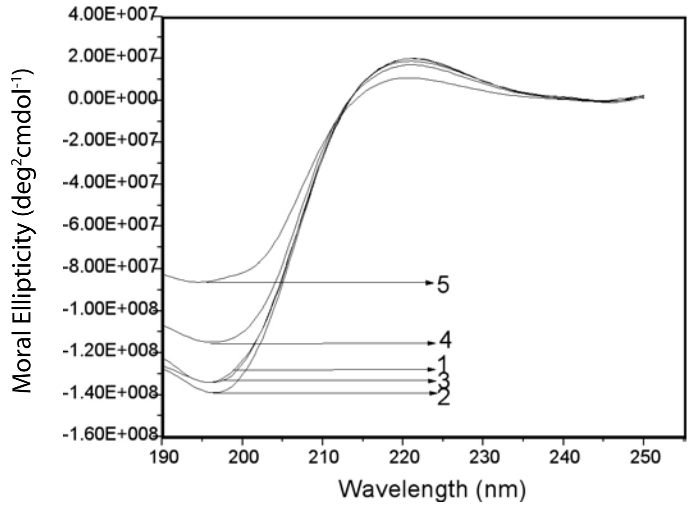

Figure 4: CD spectra of collagen (0.6 μM) tanned with zirconium which was irradiated for various time intervals. (1–Non radiated; 2-15 min; 3-30 min; 4-1h; 5- 2 h irradiation)

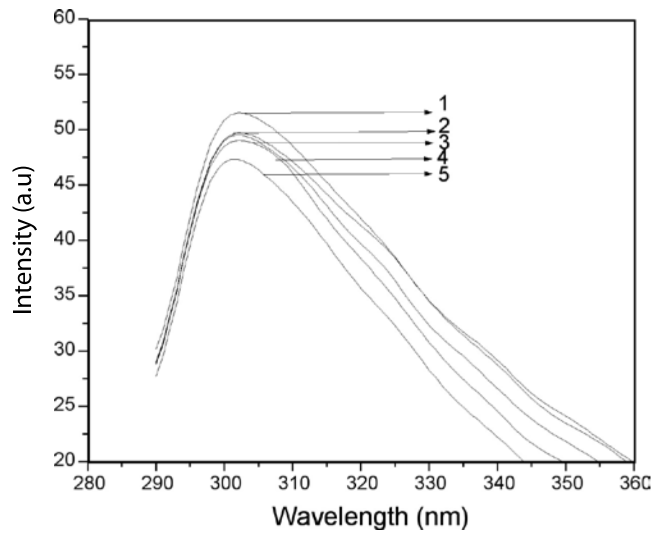

Figure 5: Effect of UV irradiation on the fluorescence spectra of native collagen solution. (1-Non radiated; 2-15 min; 3-30 min; 4-1 h; 5-2 h irradiation)

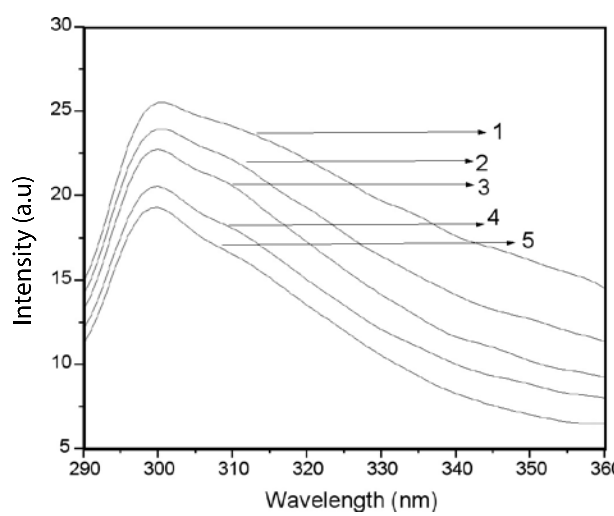

Figure 6: Effect of UV irradiation on the fluorescence spectra of collagen solution tanned with zirconium. (1-Non radiated; 2-15 min; 3-30 min; 4-1 h; 5-2 h irradiation)

Fluorescence Studies

The secondary structure analysis of collagen reveals that there are some aromatic amino acids which are involved as UV absorbing centers. Though there are various amino acids present in collagen, only a few amino acids fluoresce in collagen. Based on the change in the intensity, it has been stated that there may be a loss in the number of tyrosine residues and formation of dityrosine molecules. To study this molecular change in the structure of collagen crosslinked with zirconium, fluorescence studies have been carried out. Oxidation by UV in collagen modifies the structural and fluorescence properties of collagen components. Collagens have long been known to show evidence of fluorescence properties inside them.[24] Fig. 5 shows the fluorescence spectra of native collagen where the emission peak around 300 nm is attributed to tyrosine. The Fig. 6 shows the fluorescence spectra of collagen tanned with zirconium. From the figure, it is observed that with increasing the time of irradiation, there is a gradual decrease in the emission maxima at 300 nm when the excitation wavelength is 270 nm. However, the fluorescence intensity for zirconium tanned collagen is decreased when compared to native collagen. This can be attributed to conformational changes around the aromatic amino acid residues (tyrosine) or to structural changes in these residues themselves. It is reported that there is loss of tyrosine residue and formation of dityrosine molecule in collagen after UV irradiation.[25] It is also evident from the figure that zirconium is capable of quenching the fluorescent amino acids present in collagen.

CONCLUSION

In the present study, the effect of UV radiation on zirconium tanned collagen has been investigated. The response of native and zirconium tanned collagen to UV irradiation has been found to differ significantly. The electronic spectral studies revealed that zirconium crosslinking imparts stability to collagen against UV radiation depending on duration of irradiation. A gradual decrease in the emission maxima of crosslinked collagen on increasing the time of irradiation is observed, which is ascribed to conformational changes of the aromatic amino acids residue (tyrosine) or to the structural changes in the residues. Prolonged irradiation has been found to have a pronounced effect on secondary structure of collagen. Thus, it can be found that zirconium crosslinking imparts stability to collagen against UV irradiation depending on the duration of irradiation.

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