UV−VIS AND FT−IR SPECTRA OF ULTRAVIOLET IRRADIATED COLLAGEN IN THE PRESENCE OF ANTIOXIDANT ASCORBIC ACID

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1. INTRODUCTION

Collagen is one of the main components of skin and connective tissue [1,2], which consist of tree polypeptide α-chains wound together in a rod-like helical structure. Each molecule is 300 nm in length and 1.5 nm in diameter with a molecular weight of 300,000. The sequence of amino acid residues in the chains is very specific: glycine is every third residue in repeating sequence; Gly − X − Y and proline and hydroxyproline imino acids are X and Y, respectively, with a high probability. The molecular structure is stabilized by a net of intra- and inter-chain hydrogen bonds between − NH group of glycine and carbonyl group C = O of residues from another polypeptide chain or by hydrogen bridges with water molecules [3−5]. Collagen is the primary target of external factors such as ultraviolet (UV) radiation, which induces various physical, chemical and physical-chemical processes in living organisms. Deleterious UV light absorption is responsible for skin tanning and has been implicated as a causative factor in many kinds of skin cancer. Therefore, the study of UV radiation on a collagen molecule – to illustrate the structural changes in radiated macromolecules, to discover the possible damages after radiation and to search the effective means against the damages – attracts a special interest [6−9].

Many studies have considered the effect of UV radiation on collagen. It has been shown that the solution of collagen, after radiation, loses the ability to form natural fibrils [10]. The fluorescence observed after UV radiation is due to the presence of phenylalanine and tyrosine in this protein [11,12]. Photocrosslinking [13,14] and photodegradation [8,15] of collagen may also occur during exposure to UV radiation.

This paper is a collaboration of Georgian and Polish groups, financially supported by a NATO CLG grant. Both groups have considerable experience studying the effect of UV radiation on collagen. It has been shown that UV radiation changes the thermal helix-coil transitions of collagen samples [16,17]. In addition, the relative viscosity and fluorescence of collagen decreased, whereas the absorption / scattering of collagen solution increased during the radiation of the sample [18].

The Electron Spin Resonance (ESR) method shows that after UV radiation, the generation of primary free radicals (hydrogen atoms and acetic acid radicals) takes place in the water surrounding the acid soluble collagen. The free radicals destabilize proteins, causing the appearance of the secondary free radicals on proline and glycine residues [19,20]. Some molecular compounds, such as melanin, β-carotene and ascorbic acid, have been revealed as photo-stabilizers against photochemical changes in collagen using various techniques [18,19,21−23].

Using UV−Vis and FT−IR spectroscopy, we demonstrate that ascorbic acid, one of the effective antioxidants, acts as a photo-protective system against UV radiation.
2. MATERIALS AND METHODS

Rat tail tendon collagen was selected for investigation. Rat tails were obtained from Poznan University of Medical Sciences (Poland). Each of animals (rats) was before used in another biomedical research according the agreement done by Bioethical Commission working at Medical University in Poznan.

After washing in distilled water the tendons were dissolved in 0.04 M acetic solution [24]. Samples for investigations were prepared as in the form of solutions, as in the form of films, 0.015 mm thick. The films were dried at 35 °C and preserved at room temperature and humidity equal to 60%.

The samples (films and solutions) of pure collagen (0.9 mg / ml) and collagen containing ascorbic acid (with concentrations of 0.0015, 0.003 and 0.006 mg / ml) were irradiated under air at room temperature using a mercury lamp, Philips TUV – 30, which emits light of mainly 254 nm wavelength. The intensity of radiation during 1 h exposition was $16 J / cm^2$. The intensity of the incident light was measured using an IL 1400 A Radiometer (International Light, USA). Irradiation experiments were carried out in a quartz cuvette at a distance of 3 cm from the light source.

The UV−Vis absorption spectra of the collagen solution, before and immediately after irradiation were recorded with Shimadzu Spectrophotometer (model UV−1601 PC). Data collection and plotting were accomplished by the UVPC program and the computer data station supplied by the manufacturer.

The infrared spectra of films, before and immediately after irradiation, were recorded using spectrophotometer Mattson Genesis II (USA). Spectra were recorded by absorption mode at 4 cm$^{-1}$ interval and 16-times scanning.

3. RESULTS

3.1. UV−Vis absorption spectra

Fig. 1 shows the UV−Vis spectra of pure collagen solution before and immediately after irradiation. Due to the aromatic residues (tyrosine and phenylalanine) absorption in the range of (250 − 300) nm with a maximum at 275 nm takes place for each spectrum. This is consistent with the results obtained in prior studies [16,18]. The authors offer the following explanation: under UV irradiation, the turbidity of the solution increases due to the radiation causing changes in the structure of the collagen molecule (helix-coil transition). After 1 and 2 h irradiation, the maximum of absorption/scattering is almost the same. This fact shows that after 1 h of irradiation, collagen molecules completely change their conformational state.

Fig. 2 shows the UV−Vis spectra of non-irradiated and irradiated collagen solution in the presence of ascorbic acid, with a concentration of 0.0015 mg / ml. The conformation changes in the collagen – ascorbic acid system during irradiation occur much more slowly than in pure collagen.

In the collagen – ascorbic acid system, the difference between the maxima of absorption ($\Delta A = A - A_0$) slightly decreases (Fig. 3, curve with circles) with the increasing dose of radiation. This is in contrast to $\Delta A$ of pure collagen (curve with squares).

When the concentration of ascorbic acid was higher (0.003 mg / ml), the sample had no distinctly separate absorption with maximum at 275 nm before irradiation (Fig. 4). Under UV radiation, the absorption at (250 − 300) nm takes place again, although the process connected to the conformational changes of collagen is impeded.

Increasing the concentration of ascorbic acid (0.006 mg / ml) curves only after 45 min of irradiation have typical peak of absorption (Fig. 5) and the above mentioned process is further slowed.
Fig. 1. UV–Vis spectra of pure collagen solution before and after increasing dose of radiation (numbers indicate the times of irradiation in min).

Fig. 2. UV–Vis spectra of collagen solution in the presence of ascorbic acid (0.0015 mg / ml) before and after increasing dose of radiation (numbers indicate the times of irradiation in min).
Fig. 3. Dependence of the difference (ΔА) due to the absorbance of radiation at 275 nm of collagen (curve with squares) and collagen with ascorbic acid (curve with circles) on the time of radiation in min (А – absorbance for irradiated sample, А₀ – absorbance for non-irradiated sample).

Fig. 4. UV–Vis spectra of collagen solution in the presence of ascorbic acid (0.003 mg / ml) before and after increasing dose of radiation (numbers indicate the times of irradiation in min).
Fig. 5. UV–Vis spectra of collagen solution in the presence of ascorbic acid (0.006 mg / ml) before and after increasing dose of radiation (numbers indicate the times of radiation in min).

3.2. FT–IR spectra

Fig. 6 shows IR spectra of pure collagen films before and after UV radiation. Each spectrum represents the complex of many of the overlapping vibrational bands. Though it is not difficult to separate spectral regions of the amide A, B, I and II bands appearing at the frequencies of 3330, 3082, 1658 and 1558 cm$^{-1}$, respectively, and amide A and B are shifted to lower frequencies for irradiated samples.

The broad band at 3330 cm$^{-1}$, amide A, is due to the NH-stretching vibration. It is also due to the OH component, which confirms the active participation of water in the collagen molecule. This band is distinct from other proteins (their amide A is observed at comparatively lower frequency (3300 cm$^{-1}$), and it is more stable due to the unchangeable distance of N···O, even when the α-chains are disordered. The higher the dose of UV radiation, the lower the intensity of absorption and the more narrow the band. There is a noticeable width of amide A band in the range of (3400 − 3600) cm$^{-1}$, which sharply narrows during irradiation.

The amide B band is observed at around (3050 − 3180) cm$^{-1}$, with a maximum at 3082 cm$^{-1}$. This band also shifts to a lower wavenumber and becomes less in intensity.

The amide I band appears in the range of (1600 − 1700) cm$^{-1}$ with a maximum near 1658 cm$^{-1}$. It is produced mainly by the peptide bond C = O stretching vibration, with intensity slightly decreasing under UV radiation. This band is used for secondary-structure analysis of the polypeptide [25]. Under UV radiation, the dose does not change the position of the band.

The amide II band with a maximum at 1558 cm$^{-1}$ is connected with CNH groups, the intensity of which also decreases under UV radiation. Band position is not shifted to a lower frequency.

The data at the frequencies of the amide bands of pure collagen before and after 8 h UV radiation is shown in the table on Fig. 6.
Fig. 6. FT−IR spectra of pure collagen before (0 min) and after (30, 60, 120, 240 and 480 min) radiation.

Figs. 7 and 8 show the IR spectra of collagen films in the presence of ascorbic acid with different concentrations of and 0.003 mg / ml, respectively. Under UV radiation, the intensities of amide bands in both samples decrease as compared to non-irradiated ones, though not as sharply as it occurs in pure collagen. The amide A and B are shifted to a lower frequency at both concentrations of ascorbic acid, though far weaker than in case of pure collagen (changes in frequencies $\Delta \nu$ are less than those for pure collagen). The amide I and II bands do not change their positions (Tables on Figs. 7 and 8).
**Fig. 7.** FT–IR spectra of collagen with ascorbic acid (0.0015 mg / ml) before (0 min) and after (30, 60, 120, 240 and 480 min) radiation.
Fig. 8. FT–IR spectra of collagen with ascorbic acid (0.003 mg / ml) before (0 min) and after (30, 60, 120, 240 and 480 min) radiation.

Fig. 9 shows collected IR spectra of non-irradiated samples: pure collagen (curve 1), collagen and ascorbic acid with its 0.0015 mg / ml concentration (curve 2), and collagen and ascorbic acid with its 0.003 mg / ml concentration (curve 3). The intensities of all bands are sharply decreased in the presence of ascorbic acid, and the effect depends on the concentration of the ascorbic acid. All amide bands (A, B, I, and II) are shifted to lower frequencies in the same time (table on Fig. 9).
Fig. 9. FT–IR spectra of non-irradiated samples: collagen – curve 1, collagen with ascorbic acid (0.0015 mg / ml) – curve 2, collagen with ascorbic acid (0.003 mg / ml) – curve 3.

<table>
<thead>
<tr>
<th>Band</th>
<th>Pure collagen</th>
<th>Collagen with ascorbic acid (0.0015 mg / ml)</th>
<th>Collagen with ascorbic acid (0.003 mg / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amide A</td>
<td>3330</td>
<td>3323</td>
<td>3318</td>
</tr>
<tr>
<td>Amide B</td>
<td>3082</td>
<td>3081</td>
<td>3078</td>
</tr>
<tr>
<td>Amide I</td>
<td>1658</td>
<td>1658</td>
<td>1657</td>
</tr>
<tr>
<td>Amide II</td>
<td>1558</td>
<td>1556</td>
<td>1556</td>
</tr>
</tbody>
</table>

4. DISCUSSION

4.1. UV–Vis absorption spectra

Under UV radiation the conformational changes in collagen – ascorbic acid system occur much more slowly than in pure collagen (Fig. 3). It is likely due to the ascorbic acid’s antioxidant feature. Under UV radiation, collagen with ascorbic acid is not as sensitive as collagen alone.

The changes of UV–Vis absorbance profile in the range of (250 – 300) nm (Fig. 4 – 0 min irradiation and Fig. 5 – 0, 5, 10, 15, 30 and 45 min irradiation) might be due to the additional effect of ascorbic acid. We suggest that ascorbic acid connects to collagen with its C = O group. It is possible that OH groups of collagen molecules (mainly of tyrosine amino acid aromatic ring) combine with C = O of ascorbic acid (Scheme 1) to form a hydrogen bond. Another aromatic amino acid is phenylalanine, aromatic ring of which is like that of benzene. It is very hydrophobic and chemically reactive only under extreme conditions. That is why we exclude it from participation in
the mentioned binding reaction. Our previous studies [16,20,22] have been connected with free radicals appearing in collagen water solutions under UV radiation and evoking photodegradation of macromolecule. The aromatic amino acids found in collagen are the primary source of free electrons that form the free radicals. Ascorbic acid, a perfect antioxidant, either donates its electron to restore deleterious free radicals, thus itself becoming a non-toxic free radical, or it impedes aromatics to transit in an excited state under UV radiation, or both. In any case, it is beyond doubt that ascorbic acid changes the environment of a collagen molecule, and the effect depends of the concentration of the ascorbic acid. When increasing the dose of radiation, more molecules of ascorbic acid are hindered, and the antioxidant effect is diminished accordingly.

4.2. FT–IR spectra

Intensities of FT–IR bands have changed under UV radiation. The higher the dose of radiation, the lower the degree of intensities of all bands (amide A, amide B, amide I and amide II) as compared to those of the non-irradiated collagen sample (Fig. 6).

In a previous study [26], it was suggested that under UV radiation, the changes in the amide A and B bands indicate the photodegradation of collagen along its main chains with scission of −CH₂−N= and =CH₂ bonds.

There is a noticeable width of the amide A band in the range of (3400 – 3600) cm⁻¹, which sharply narrows during irradiation. It is likely due to the breaking of N−H···O=C inter-chains hydrogen bond as well as losing the bonding water in collagen.

In the presence of ascorbic acid, the intensities of the amide bands of collagen also decrease under irradiation (Figs. 7 and 8), though much more slightly than it occurs for pure collagen. Positions of the amide A and B bands shift to lower frequencies, though in this case, changes in frequencies ∆ν are less (see tables on the Figs. 7 and 8) than those for pure collagen (table on Fig. 6). When we have IR spectra of the non-irradiated collagen and the collagen – ascorbic acid system (Fig. 9), intensities of all bands sharply decrease in the presence of ascorbic acid, and the effect depends on the concentration of antioxidant. The reduction of the bandwidth of amide A is presumably due to the restriction of conformational freedom of macromolecules in the presence of ascorbic acid. All amide bands (A, B, I, and II) are shifted to lower frequencies at the same time (table on the Fig. 9). The most remarkable is the amide A band, the shift of which might be due to the formation of a hydrogen bond between N−H group of peptides and C=O group of ascorbic acid (Scheme 1).

**Scheme 1.**
Results suggest that ascorbic acid increases the photo-stability of the collagen molecule; and the ascorbic acid interacts with the collagen macromolecule as well.

5. CONCLUSIONS

Structural changes in the collagen molecule occur under UV radiation. Ascorbic acid increases the photo-stability of the collagen molecule, and the molecule becomes less sensitive to the UV radiation. Interaction between collagen and ascorbic acid possibly takes place as well.

ACKNOWLEDGMENT

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REFERENCES