SPECTROSCOPIC DETERMINATION OF COLLAGEN CROSS-LINKS

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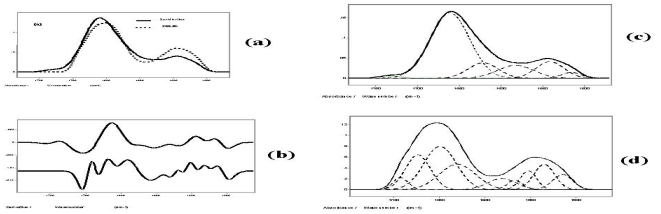
INTRODUCTION: Type I collagen's chemistry varies from tissue to tissue because of cellular & extracellular posttranslational modifications. The most distinct feature of type I collagen in mineralized tissues is its cross-linking chemistry and molecular packing structure. Fourier transform infrared (FTIR) spectroscopy has been used previously for analyzing three-dimensional structures of collagen and other proteins in solution. Vibrational bands characteristic of peptide groups and side chains provide information on protein structures. Among these, the Amide I band (peptide bond C=O stretch ~1650 cm^{-1}) is especially sensitive to secondary structures. In such studies, information on protein structures is extracted from broad envelopes consisting of component bands arising from the Amide I modes of various secondary structures by applying a technique of resolution enhancement such as Fourier self-deconvolution, second derivative spectroscopy, and difference FTIR. In the present study, a series of collagens obtained from bovine bones and purified cross-linked peptides were analyzed by means of FTIR as KBr pellets (≈ 0.5% wt/wt), to derive spectroscopic parameters describing pyridinoline and DHLNL. This information is used to evaluate detailed collagen cross-link patterns in biopsied tissues.

MATERIALS & METHODS: Collagen and collagen purified cross-linked peptides were prepared as described [1]. Collagen from bovine femoral bone (4mo and 9yr- old) was used.. Collagen from the 4mo age group was used for isolation and spectroscopical characterization of the major cross-linked tryptic peptides. Collagen from the 9yr group was used 1. undigested to compare the spectroscopic properties of it to these of the 4mth- group (also undigested) and 2. to isolate the major pyridinoline cross-linked peptide and perform pyridinium ring cleavage by UV-photolysis [ref] for spectroscopic analysis. KBr pellets of the various samples were analyzed by means of FTIR using a BioRad FTS40 spectrometer (BioRad, MA). Spectra were obtained at a spectral resolution of 4 cm^{-1}. The spectra were baseline corrected in the Amide I and II spectral area and water vapor contribution subtracted according to the criteria published elsewhere [2]. Second derivative spectra were calculated [ref] and the resulting peak positions were utilized as initial input (of Gaussian type) in the curvefitting routine (Grams32, Galactic Software, NH). The output of this analysis was expressed as position of underlying band and relative % contribution.

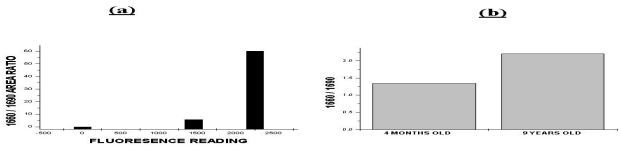
RESULTS: The baseline-corrected, area-normalized FTIR spectra of pyridinoline (solid line) and DHLNL (dashed line) purified cross-linked

FIGURE 1



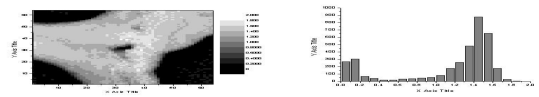
collagen peptides are shown in Fig 1a. Subtle yet definite differences exist between the two. Their respective calculated second derivative spectra are shown in Figure 1b. One readily observed difference is that DHLNL (bottom trace) has a peak ≈ 1690 cm^{-1} not evident in the second derivative spectrum of pyridinoline. It also lacks a maximum ≈ 1660 cm^{-1}, unlike in the case of pyridinoline. Figs 1c and 1b show the spectra of pyridinoline and DHLNL, respectively, resolved to their underlying bands as determined by second derivative spectroscopy and curvefitting techniques. These results suggest that it is feasible to spectroscopically distinguish between these two types of collagen cross-links. To further verify this hypothesis, purified pyridinoline cross-linked peptides were subjected to degradation by UV light. The amount of the remaining intact pyridinoline bond was determined by fluorescence spectroscopy. Samples were also analyzed by FTIR, and 1660/1690 ratios calculated. Fig 2a plots the spectroscopically derived ratio against the fluorescence readings. This ratio is sensitive to amount of pyridinoline cross-link bond present. To verify that this spectroscopically defined ratio is capable of representing the pyridinoline/DHLNL relative amounts in type I collagen molecules as well as in isolated purified cross-linked peptides, bovine collagen was spectroscopically analyzed from animals of two different ages (4

FIGURE 2



mo and 9yr (Fig2b). According to this ratio, collagen from the older animals contains a higher amount of pyridinoline relative to DHLNL, consistent with results obtained biochemically [1]. The parameters that were developed may be readily applied to FTIR Imaging analyses, thus permitting the monitoring of collagen cross-link patterns in 400x400 um^2 areas of thin tissue sections from biopsies at a spatial resolution of 10 um. An example is given in Figure 3, where parts of the trabecular bone in a normal

FIGURE 3



human (56 yr old male) iliac crest biopsy was analyzed for collagen cross-link spatial patterns. The output of this analysis is color-coded, but for the purposes of this abstract it was converted to grey-scale (black=0, white=maximum). The data may be also converted into a histogram, as shown in Fig 3, thus providing a measure of parameter value distribution. As may be seen the lower values occur at the periphery of the trabeculae while the higher ones in the geometrical center, suggesting a progressive increase in the relative amount of pyridinoline with respect to DHLNL from the outside periphery towards the center (where the "oldest" bone lies).

DISCUSSION: The results of the present show that it is feasible to monitor the relative amount of pyridinoline/DHLNL cross-links spectroscopically. This is of great importance since similar analyses may be employed using FTIR Imaging, a technique that allows the analysis of 400 x 400 um^2 areas in thin tissue sections with a spatial resolution of 10 um, thus permitting the establishment of the relative amount of these two collagen cross-links at the ultrastructural level.

REFERENCES: 1)Yamauchi*:* in *Calcium and Phosphorus in Health and Disease*1996, CRC Press: NY 127, 2) Dong, et al., Biochem, 1990. **29**. 3303.

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