ISOLATION AND CHARACTERIZATION OF A FLUORESCENT MATERIAL IN BOVINE ACHILLES TENDON COLLAGEN

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SUMMARY A fluorescent material in bovine achilles tendon collagen was isolated and characterized by ultraviolet spectroscopy, fluorescence spectroscopy and nmr spectroscopy. The data suggest that the compound is a 3-hydroxypyridinium derivative with three amino acid side chains. The name "pyridinoline" is proposed. Pyridinoline is a novel type crosslink of collagen.

It is known that collagen from various sources contains a visibly fluorescent material (1,2) and that the concentration of the material increases with age (1). However, the fluorescent material has not been characterized yet. This paper describes the isolation and characterization of a fluorescent material in bovine achilles tendon collagen. The compound is a 3-hydroxypyridinium derivative with three amino acid side chains.

MATERIALS AND METHODS

Bovine achilles tendon collagen was obtained from Worthington Biochemical Corp., U.S.A.(lot #36E649). In some experiments, the collagen supplied by Dr. T. Fujii, Nippi Co., Japan, was used. A typical example of the isolation of the fluorescent compound is described below. Collagen (2 g) was hydrolyzed with 6N HCl (80 ml) at 110°C for 24 hrs in sealed tubes. The solution was dried up in vacuo and the hydrolysate was taken up in water and applied on a P-cellulose column (H^+ form, 1.8 x 30 cm). Elution was performed with a linear gradient, starting with 400 ml of water in the mixing chamber and 400 ml of 0.5N HCl in the reservoir and fractions (15 ml each) were collected. Fractions containing the fluorescent compound (Fr. no.41-46) were combined and evaporated in vacuo. The residue was taken up in water and applied on a small column of P-cellulose (H^+ form). After washing with water, the fluorescent compound was eluted with 2N NH_4OH. The compound was further purified by passing through a DEAE-cellulose column (acetate form, 1.2 x 10 cm) and a Sephadex G-10 column (1.2 x 30 cm).

The fluorescence spectra were measured with Shimazu RF-502 corrected recording spectrofluorophotometer. ^1H-nmr spectrum was measured with JEOL JMN-FX60 at 59.80 MHz at 27°C in D_2O: pulse width 16 μs, repetition time 2.0 s, spectral width 1057 Hz/4096 points. Absorption spectrum was recorded by Fourier transformation of FID which were accumulated 20 times and then the real spectrum was obtained by 1000 times repetition of the same process during which proton signal in D_2O was overflowed. Paper electrophoresis was performed with Camag high voltage electrophoresis system. The amount of amino

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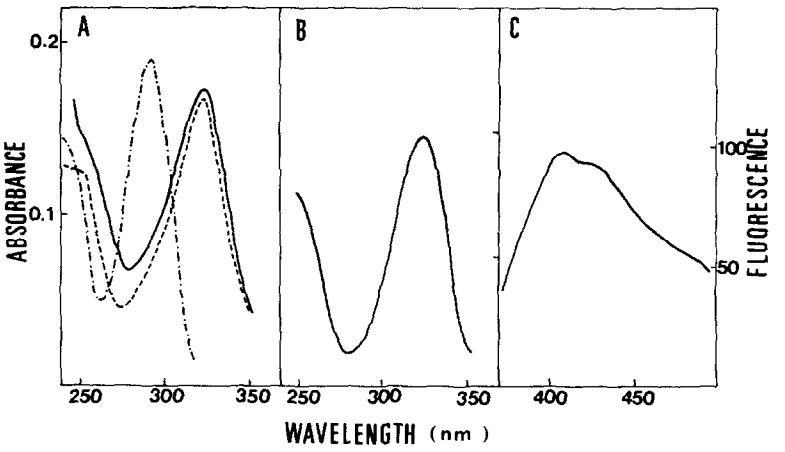

Fig.1. (A) Ultraviolet absorption spectra in 0.1N HCl (—·—·—), in 0.1M potassium phosphate buffer, pH 7.4 (·····) and in 0.1N NaOH (———). (B) Activation spectrum in 0.02M potassium phosphate buffer, pH 7.4. Fluorescence at 400 nm. (C) Fluorescence spectrum in 0.02M potassium phosphate buffer, pH 7.4. Activation at 325 nm.

groups was estimated by trinitrophenylation according to Okuyama and Kasai (3). Partial dinitrophenylation was performed according to Andersen (4).

RESULTS AND DISCUSSION

The isolated compound gave a single fluorescent and ninhydrin-positive spot on paper chromatograms in the solvent systems of n-butanol-acetic acid-water (4:1:2), phenol-water (4:1) and t-butanol-formic acid-water (14:3:3). Approximately 1 mg of the compound was obtained from 2 g of collagen.

The ultraviolet absorption spectra of the compound are shown in Fig. 1A. The absorption maximum is at 295 nm in acid solution and at 325 nm in neutral and alkaline solution. As shown in Table 1, the spectra of the compound closely resemble those of 3-hydroxy-N-methylpyridines, particularly N-methyl-pyridoxine. The spectra differ from those of other 3-hydroxypyridines in that the absorption maximum is unchanged when the pH is increased from neutral to alkaline. 2- or 4-hydroxypyridines are known to exist predominantly as pyridone forms and their spectra remain constant irrespective of the pH of the solution (6). The pH at which the bathochromic shift occurs is about 4.0, which is one pK_a unit lower than the pK_a value of the hydroxyl group of

TABLE 1

ULTRAVIOLET ABSORPTION SPECTRA OF SOME HYDROXYPYRIDINES

Compound	Absorption maximum			Reference
	acid	neutral	alkaline	
Unknown compound	295	325	325	
3-Hydroxypyridine	283	313	298	5
N-Methyl-3-hydroxypyridine	288		322	5
2-Hydroxypyridine	295		295	6
N-Methyl-2-hydroxypyridine	293	297	297	
Pyridoxine	291	324	310	5
N-Methylpyridoxine	295	330	330	5

pyridoxine but approximately equal to that of pyridoxylserine (5). The pK_a values of phenols are much higher than 4 and, therefore, the possibility that the compound is a derivative of phenols is unlikely.

The excitation and fluorescence spectra of the compound in neutral solution are shown in Fig.1B and 1C. The excitation maximum is at 325 nm and the fluorescence maximum is near 410 nm. These spectra resemble those of pyridoxine in neutral solution (7) and those obtained with intact collagen (1,2).

The nmr spectrum of the compound in D_2O is shown in Fig.2. A sharp singlet at 7.62 (1 H) corresponds to a pyridine ring proton. The position of the ring proton can be assigned not to be α by comparison with the chemical shift of α proton of pyridoxine hydrochloride (8.13) and that of pyridoxine methiodide (8.36) in D_2O. The other absorptions correspond to methine (3.6 - 4.1, 4H, m), methylene attached to pyridinium nucleus (3.20,d and 2.5 - 2.9, m, 2~3H) and aliphatic methylene groups (1.5 - 2.2, 4H, m). The signal of methylene attached to N^+ overlapped those of water and could not be observed. These assignments are based upon the nmr spectra of desmosine (8) and hydroxyaldol-histidine (9).

The results indicate that the compound is a 3-hydroxypyridinium derivative with amino acid side chains. Assuming that the compound has the same molar absorption coefficient at 295 nm as that of N-methylpyridoxine (8300 in acid solution, ref.5) and the color yield for each amino group in trinitro-

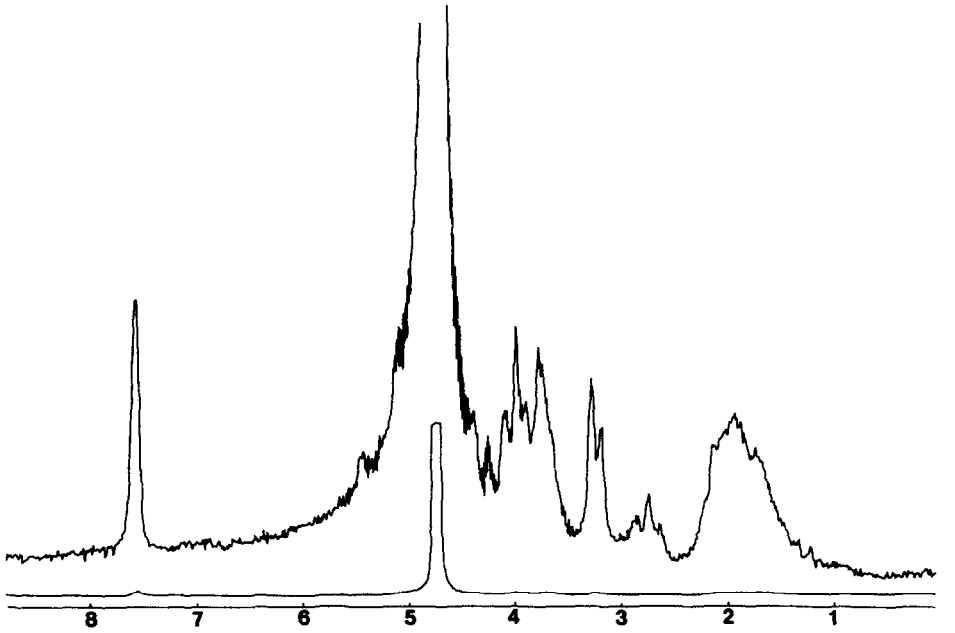

Fig.2. NMR spectrum.

phenylation (3) is the same as that of leucine, the number of amino group in the compound was estimated to be 2.87 per molecule. This result is consistent with the result of partial dinitrophenylation experiment. When the compound was partially dinitrophenylated and then subjected to paper electrophoresis in a strongly acid solvent (4), it gave three yellow spots and one fluorescent spot, indicating that the compound has three amino groups per molecule. The number of carboxyl group could not be determined. However, the mobility of the compound in paper electrophoresis at pH 5.6 indicated that it is neutral at this pH and therefore, the number of carboxyl group may be the same as that of amino group.

The molecular weight of the compound was roughly estimated by gel filtration on a Sephadex G-15 column with 0.1M phosphate buffer, pH 7.4,as a solvent. Calibration of the column by filtration of pyridoxine, pyridoxylalanine and phosphopyridoxylalanine gave an apparent molecular weight of about 400 to the present compound.

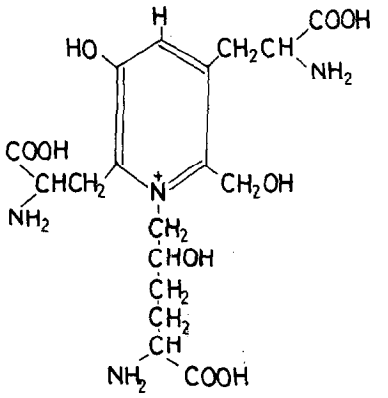

Fig.3. A possible structure.

Now it can be concluded that the pyridinium ring of the compound has one proton, one hydroxyl group, three amino acid side chains and one unidentified substituent group. It is known that various crosslinks in collagen and elastin are derived from lysyl and hydroxylysyl residues (10). For example, desmosine, a tetrafunctional crosslink containing pyridinium ring, has been shown to arise from oxidative condensation of four lysyl residues (10). If we can assume, by analogy, that the compound is derived from three hydroxylysyl residues, one of the most possible structures is proposed as shown in Fig.3. This structure is compatible with all the spectral data and molecular weight data (calcd. 445), except that the number of proton of methylene attached to the nucleus obtained by integral of the nmr peaks (2~3 H) is not coincident with the calculated value (4H). The nmr spectrum of N-methylpyridoxine indicates that the signals of protons of CH_2OD attached to pyridine ring lie close to those of water(δ = 5.18 and 4.85). Therefore, the presence of this group on the pyridinium ring of the compound could not be confirmed by the nmr spectrum.

The structure indicates that the compound is a novel type crosslink of collagen. Although the complete structure remains to be elucidated, the compound is characterized by 3-hydroxypyridinium ring and we propose the name

"pyridinoline" for this compound.

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