Vol. 93, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1980 Pages 948-953

EVIDENCE FOR NATURAL EXISTENCE OF PYRIDINOLINE CROSSLINK IN COLLAGEN

Daisaburo Fujimoto

Hamamatsu University School of Medicine
Hamamatsu 431-31, Japan

Received March 3, 1980

SUMMARY Pyridinoline is a fluorescent crosslinking amino acid isolated from collagen. Recently it was claimed that this material is an artefact produced from contaminating proteins during acid hydrolysis. However, in our hands, bovine tendon collagen could not be depleted of pyridinoline by the suggested treatments. A peptide which had the same fluorescence properties as those of pyridinoline could be isolated from enzymic digests of collagen. After acid hydrolysis, presence of pyridinoline in the peptide could be demonstrated on amino acid analysis. The composition of the peptide suggests that it originates from the specific regions of collagen molecule. These results clearly indicate the existence of pyridinoline in collagen in vivo.

Pyridinoline is a fluorescent crosslinking amino acid isolated from acid hydrolysate of collagen (1). It was shown by nmr spectroscopy and other techniques to be a 3-hydroxypyridinium derivative with three amino acid side chains (1,2). It abounds in collagen of mature animals (3,4).

Recently Elsden et al. reported that collagen was depleted of pyridinoline by mild washing procedures or pepsin digestion, and claimed that pyridinoline is an artefact produced from contaminating proteins during acid hydrolysis (5).

However, we found that pyridinoline could not be removed by the treatments suggested by Elsden et al., using more specific assay method. Moreover, a peptide which had the same fluorescence characteristics as those of pyridinoline could be isolated after enzymic hydrolysis of collagen. These results clearly demonstrate that pyridinoline is not an artefact but occurs in collagen in vivo.

MATERIALS AND METHODS

Bovine tendon collagen (type I) was obtained from Sigma Chemical Co., St. Louis.

Pepsin digestion and washing with urea-acid were performed as described by Elsden et al (5).

The protein was hydrolyzed with 6N HCl at 110°C for 24 hr in a sealed tube. The pyridinoline content in the hydrolysate was determined by fluorescence assay after P-cellulose chromatography (6). A sample was

0006-291X/80/070948-06$01.00/0
Copyright © 1980 by Academic Press, Inc.
All rights of reproduction in any form reserved.

applied to a P-cellulose column (H^+form, 1.2 x 8 cm) and elution was performed with a linear gradient formed from 60 ml of water and 60 ml of 0.5M HCl. The fractions (3 ml each) were collected and the fluorescence at 395 nm was measured with excitation at 295 nm. Pyridinoline was eluted between fraction nos.26 and 30 and its amount was estimated by comparison of its fluorescence intensity with that of a known amount of pure pyridinoline.

For thermolysin digestion, collagen (2 g) was suspended in 180 ml of 0.05M tris-5 mM $CaCl_2$, pH 7.6, and heated at 80°C for 20 min and then mixed with 10 mg of thermolysin (Seikagaku Kogyo, Tokyo). The mixture was incubated at 40°C for 8 hr, and then centrifuged at 10,000 x g for 20 min. The supernatant was applied to a CM-Sephadex C25 column (H^+form, 1.8 x 20 cm). Elution was performed using a linear gradient formed from 0.7 l of water and 0.7 l of 0.7M NaCl. Peptide concentration was estimated by measuring absorbance at 230 nm and pyridinoline concentration was estimated by measuring fluorescence at 395 nm with excitation at 295 nm. The fractions marked with a bar in Fig.2A were pooled, concentrated under reduced pressure and desalted by gel filtration on a Sephadex G10 column. The desalted peptides were applied to a Sephadex G25 column (1.2 x 130 cm) and eluted with 0.1M acetic acid. The fractions marked with a bar in Fig.2B were collected, dried up under vacuum, taken up in 1 mM sodium citrate buffer, pH 3.6, and applied to a P-cellulose column (1.2 x 16 cm) , which had been equilibrated with the same buffer. Elution was performed at 42°C using a linear gradient formed from 300 ml of 1mM sodium citrate buffer, pH 3.6, and 300 ml of the same buffer containing 0.6 M NaCl.

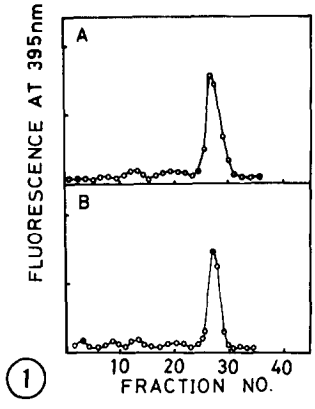
For N-terminus analysis, the peptide was dinitrophenylated and hydrolyzed and DNP-amino acids were separated by two-dimentional chromatography on a microcrystalline cellulose plate employing the solvent systems of n-butanol saturated with 0.1% NH_3 and 1.5 M sodium phosphate, pH 6.0.

High voltage paper electrophoresis was performed on a Camag HVE system at 40 volt/cm for 60 min in 5% formic acid-15% acetic acid. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed essentially according to Weber and Osborn (7), but the gel contained 15% acrylamide.

Amino acid analysis was performed using Jeol 6AH amino acid analyzer.

RESULTS

Elsden et al. reported that untreated bovine tendon collagen contained pyridinoline but, after washing with 4M urea containing 0.5M acetic acid at 60°C for 8 hr, the collagen contained no detectable amount of pyridinoline (5). However, as shown in Fig.1, pyridinoline was found in both untreated and urea-acid treated collagens. The contents of pyridinoline in untreated and treated collagens were calculated to be 0.14 and 0.19 mole/100 mole hydroxyproline, respectively. Elsden et al. reported also that neither pepsin-solubilized nor pepsin-insoluble collagen contained appreciable amounts of pyridinoline(5). However, we found that both collagens contained significant amounts of pyridinoline; the contents in pepsin-solubilized and insoluble collagens were 0.11 and 0.20 mole/100 mole hydroxyproline, respectively. These results



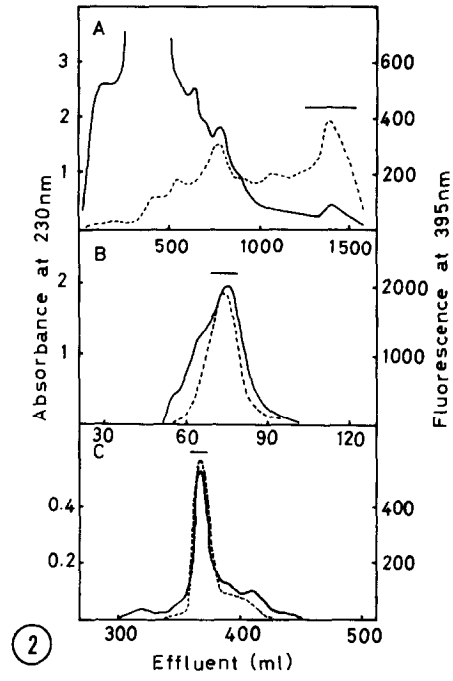


Fig.1 P-cellulose chromatography of the hydrolysates of collagen washed with 4M urea-0.5M acetic acid at 60°C for 8 hr (A) and untreated collagen (B). Each sample contained 11.7 μmoles of hydroxyproline. Intensity of fluorescence is expressed in arbitrary units.

Fig.2 Isolation of a fluorescent peptide from thermolysin digest of bovine tendon collagen. Absorbance at 230 nm (———) and fluorescence at 395 nm (-----) were measured. Details of the procedures are described in the text. (A) CM-Sephadex chromatography, (B) Sephadex G 25 Chromatography, (C) P-cellulose chromatography.

indicate that collagen could not be depleted of pyridinoline by the treatments suggested by Elsden et al. (5). These treatments rather preferentially solubilized less-crosslinked collagen, resulting in an increase in the pyridinoline content in the insoluble collagen.

In a previous study, a fluorescent peptide was isolated from pronase digests of bovine tendon collagen, however, the peptide could not be characterized sufficiently (6). In this study, bovine collagen was digested with thermolysin and peptides were fractionated as shown in Fig.2. The final peptide preparation gave a single fluorescent and ninhydrin-positive spot on high voltage paper electrophoresis and gave one band on SDS polyacrylamide gel electrophoresis. It migrated a little faster than insulin but slower than

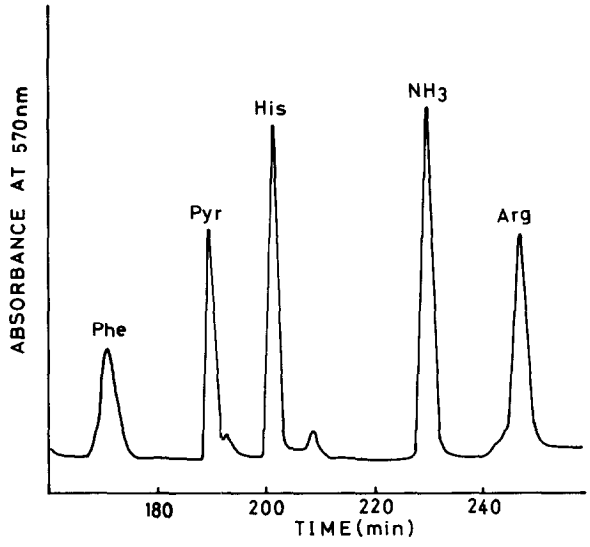


Fig.3 Amino acid analysis of acid hydrolysate of the fluorescent peptide. Pyr denotes pyridinoline.

reduced insulin peptides, suggesting that the molecular weight was approximately 4000. N-terminal analysis indicated that the major N-terminal amino acid was phenylalanine and the minor one was alanine. The apparent chain length(i.e. total amino acids/ N-termini) was found to be approximately 13.

The excitation and fluorescence maxima of the peptide were at 295 nm and 395 nm in 0.1M HCl, and at 325 nm and 395 nm in 0.1N NaOH, respectively, which were identical with those of isolated pyridinoline (1,6). This clearly indicates that pyridinoline occurs in vivo and does not represent an artefact from the hydrolysis procedure. After hydrolysis, the presence of pyridinoline in the peptide could be demonstrated on amino acid analysis (Fig.3).

The amino acid composition (Table 1), N-termini and size of the peptide and the specificity of thermolysin (8) suggest that the peptide is triple-chained and two chains originate from the sequence ^{1035}Phe-Leu-Pro-Gln-Pro-Pro-Gln-Gln-Glx-Hyl-Ala-His-Asp-Gly-Gly-Arg-^{1051}Tyr in α1 chain and one chain originates from the sequence ^{97}Ala-Gly-Leu-Hyp-Gly-Met-Hyl-Gly-His-Arg-Gly^{107} in α1 (9). Pyridinoline may arise from three hydroxylysine residues (2). The observed composition agrees with the values calculated from the proposed sequences,

TABLE 1

Amino acid composition of fluorescent peptide

Amino acid	Relative content	Nearest integer	Amino acid	Relative content	Nearest integer
Hyp	0.5	1	Leu	2.6	3
Asp	3.1	3	Tyr	0.9	1
Glu	8.1	8	Phe	1.9	2
Pro	6.3	6	Pyridinoline*	0.9	1
Gly	7.9	8	His	3.0	3
Ala	2.9	3	Arg	3.2	3
Met	0.7	1	Total	42.0	43

*The molar color yield was found to be about 250% of that of leucine.

except that one additional aspartic acid is present. This might be ascribed to the difference between skin collagen and tendon collagen. The C-terminal tyrosine may have been incompletely removed by thermolysin digestion. The sequence 1035-1051 is in the C-terminal, non-helical region and the sequence 97-107 is in the helical region near N-terminus of collagen molecule (9).

DISCUSSION

The results described in this paper demonstrate that pyridinoline is not an artefact but occurs in collagen *in vivo*, in contrast to the results obtained by Elsden et al. (5). The discrepancy might be ascribed to the difference of the specificity and sensitivity of the assay methods. Elsden et al. assayed pyridinoline with whole protein hydrolysate on amino acid analyzer (5). Since the proportion of pyridinoline is very small, in our experience, it was difficult to assay it correctly by direct analysis of whole hydrolysate on amino acid analyzer using ninhydrin reaction. Pyridinoline has a characteristic fluorescence and, therefore, the fluorescenc measurement may be more specific and sensitive.

Elsden et al. also claimed that they could not detect hydroxylysine in the heat degradation products of pyridinoline (5) We have observed the formation of hydroxylysine in a low yield when pyridinoline was heated at 110°C in water (2). Under our conditions, most of pyridinoline was recovered unchanged (2), whereas pyridinoline disappeared after the reaction in the experiment by Elsden et al. (5). The reaction conditions may be critical,

and further studies are needed to elucidate the chemistry of heat degradation of pyridinoline. However, incorporation of radiolabelled lysine into pyridinoline in vivo (4) and in vitro (Siegel, R.C., Fu, J., Uto, F. and Fujimoto,D., to be published) have confirmed that pyridinoline derives from lysine.

The amino acid composition of the isolated peptide suggests that pyridinoline unites two C-terminal, non-helical regions and one helical region near N-terminus of collagen molecules. It is known that dehydro-dihydroxylysinonorleucine, a bifunctional crosslink which abounds in immature collagen, unites one C-terminal non-helical region and one helical region near N-terminus (10). The location of pyridinoline is compatible with the proposed product-precursor relationship between pyridinoline and dehydro-dihydroxylysinonorleucine (4).

ACKNOWLEDGEMENTS

I thank Prof. Y. Nagai, Tokyo Medical and Dental University for amino acid analysis. This work was supported in part by a grant from the Ministry of Welfare, Japan.

REFERENCES

1. Fujimoto, D., Akiba, K. and Nakamura, N. (1977) Biochem. Biophys. Res. Commun. 76, 1124-1129
2. Fujimoto, D., Moriguchi, T., Ishida, T. and Hayashi, H. (1978) Biochem. Biophys. Res. Commun. 84, 52-57
3. Moriguchi, T. and Fujimoto, D. (1978) J. Biochem. (Tokyo) 84, 933-935
4. Eyre, D. R. and Oguchi, H. (1980) Biochem. Biophys. Res. Commun. in press
5. Elsden, D. F., Light, N.D. and Bailey, A. J. (1980) Biochem. J. 185, 531-534
6. Fujimoto, D. and Moriguchi, T. (1978) J. Biochem. (Tokyo) 83, 863-867
7. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
8. Heinrikson, R. L. (1977) Method. in Enzymol. 47E, 175-189
9. Piez, K. (1976) in Biochemistry of Collagen (Ramachandran, G. N. and Reddi, A. H. eds.) pp 1-40, Plenum Press, New York
10. Eyre, D. R. and Glimcher, M. J. (1973) Biochem. Biophys. Res. Commun. 52 663-671