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LOCATION OF THE INTERMOLECULAR CROSS-LINKS IN BOVINE DENTIN COLLAGEN, SOLUBILIZATION WITH TRYPSIN AND ISOLATION OF CROSS-LINK PEPTIDES CONTAINING DIHYDROXYLYSINONORLEUCINE AND PYRIDINOLINE

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SUMMARY [^{3}H]$NaBH_4$ reduced bovine dentin collagen was denatured at 60°C for 1 hr and then digested with trypsin. The digest was still substantially insoluble suspension, but it was found that 99% of dentin collagen can be solubilized if the digest was heated again at 60°C for 15 min. Two cross-linked tryptic peptides were isolated from this digest by sequential chromatographies on Sephadex G50, phosphocellulose and DEAE-cellulose column. One isolated peptide was characterized as a 59 residue cross-linked peptide including one residue of dihydroxylysinonorleucine and the other was 103 residue including one residue of pyridinoline. The amino acid compositions were consistent with the identification of the 59 residue peptide as the sequence in α1-CB4-5 (76-90) linked to the sequence in α1-CB6 (990-23^C), and the 103 residue peptide as the sequence 76-90 linked to two of the sequence 990-23^C. These results strongly support the previously proposed precursor-product relationship between dihydroxylysinonorleucine and pyridinoline.

Dentin collagen is known to be highly insoluble with the solvents commonly used to extract the other collagens. The highly insoluble nature of this collagen has been ascribed to the unique cross-link distribution (13,14). Characterization of the reducible cross-link pattern in dentin collagen showed that the most abundant cross-link in this collagen was dihydroxylysinonorleucine (DHLNL) (13), but quantitation of DHLNL and hydroxylysinonorleucine (HLNL) in dentin collagen revealed that the contents were not remarkably high compared with the other collagens (12). Therefore, some other forms of cross-link probably the non-reducible metabolite of DHLNL was believed to be responsible to the unique stability of this collagen (14). Recently, Scott (14) has isolated the peptides which were cross-linked between α2-CB4 and α2-CB1 or α1CB-0,1 via the as yet unknown cross-link. However, any other

Abbreviations: DHLNL, dihyroxylysinonorleucine; HLNL, hydroxylysinonorleucine

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location of the known cross-link in dentin collagen has not been elucidated in spite of the previous efforts (9).

Pyridinoline, a novel cross-link which is non-reducible and fluorescent, was detected in cartilage, tendon and dentin collagen, first by Fujimoto and his colleagues (6), and later by Eyre and Oguchi (4). In this paper we describe the isolation of the peptides containing DHLNL and pyridinoline, and assign their location in the collagen fibrils.

MATERIALS AND METHODS

Dentin was obtained from unerupted bovine incisors (about two year-old) and powdered in a stainless steel mortar and pestle, under liquid nitrogen. Dentin powder was demineralized with 0.5M EDTA, 0.05M Tris-HCl, pH 7.5, washed with deionized water and lyophilized. The dentin collagen was suspended in 0.154M Bes buffer, pH 7.4 and reduced with [^{3}H]$NaBH_4$ (250 mCi/mmol) as previously reported (9).

The reduced collagen was suspended in 0.05M Tris-HCl, pH 7.4, 0.01M $CaCl_2$ and heated at 60°C for 1 hr with stirring. The suspension was cooled to 37°C and digested with trypsin (1% w/w) (TPCK-treated, Worthington) for 15 hr at the same temperature. The mixture was again heated to 60°C for 15 min. Dentin collagen was completely solubilized except the slight amount of turbidity which was identified as the calcium-precipitable phosphoprotein (11). The mixture was cooled again to 37°C and retreated with trypsin (0.5% w/w) for 3 hr. The supernatant was lyophilized after centrifugation.

Portion of the dried tryptic peptides was dissolved in 0.05M triethylamine-formate, pH 8.9 and applied to a Sephadex G50 superfine column (5.05 x 100 cm) equibrated with the same solvent. The fraction containing the largest amount of radioactivity indicated by the bar (peak 3b in Fig. 1) and the fraction containing the largest amount of fluorescence (excited at 325 nm, analyzed at 395 nm) (peak 2 in Fig. 1) were collected, lyophilized and dissolved in 2M guanidine-HCl, 0.05M Tris-HCl, pH 7.4. Both fractions were applied to a Sephadex G50 superfine column (2.6 x 94 cm) using the same 2M guandine solution (Fig. 2A and 3A). One of the radioactive peaks (indicated by the bar in Fig. 2A) and the fluorescent peak in Fig. 3A were desalted on P-2 column, lyophilized and dissolved in 1 mM lithium formate buffer, pH 3.8.

These two fractions were applied to a phosphocellulose column (Whatman P-1, 2.5 x 10 cm) that had been equilibrated with the same buffer. The column was eluted at 40°C with linear gradient using 400 ml of 1 mM lithium formate pH 3.8 and 400 ml of the same buffer containing 0.5M LiCl (Fig. 2B and 3B).

Fractions containing the largest amount of radioactivity and fluorescence (peaks indicated by the bars in Fig. 2B and 3B respectively) were collected, desalted on P-2 column, lyophilized and further fractionated on DEAE-cellulose column (Whatman DE 52, 1.6 x 10 cm) which had been previously equilibrated with 0.01M Tris-HCl, pH 8.2 buffer. Separation was performed first by eluting with 50 ml of the column-equilibration buffer and the linear gradient consisting of 200 ml of 0.01M Tris-HCl, pH 8.2 and the same buffer containing 0.2M NaCl (Fig. 2C and 3C). The column temperature was 42°C. The peaks indicated by the bars in Fig. 2C and 3C were collected as the final preparations.

Amino acid analysis were performed using a Hitachi 835 amino acid analyzer, after the samples were hydrolyzed with 6N HCl at 110°C for 24 hr in the sealed evacuated tube. Cross-link analysis were carried out as described (12). Pyridinoline was kindly given by Dr. Fujimoto.

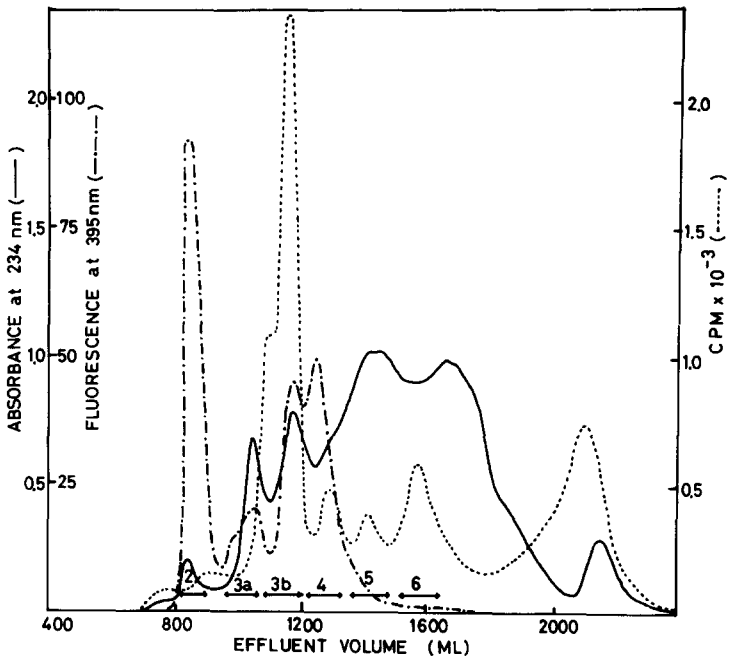

Fig. 1. Sephadex G50 superfine chromatography of the trypsin digest of dentin collagen. Fraction 2 and 3b indicated by the bars were collected for further purifications.

RESULTS

Major radioactive peak in Fig. 1 (peak 3b) contained almost solely DHLNL as the reducible cross-link on cross-link analysis. Major fluorescent peak in Fig. 1 (peak 2) showed the maxima of excitation and fluorescence at 325 nm and 395 nm at pH 8.9, and 295 nm and 395 nm at pH 3.8, respectively, which were identical with those of isolated pyridinoline (6), and pyridinoline was identified in this peak on amino acid analysis. These two peaks were rechromatographed on the calibrated Sephadex G50 superfine column eluted with 2M guanidine. The former was eluted as a single peak with molecular weight of about 10,000 (Fig. 3A), and the latter was separated into the two peaks with molecular weight of about 7,000 and 4,000 respectively (Fig. 2A). The peaks indicated by the bars in Fig. 2A and 3A were further purified on a phosphocellulose (Fig. 2B and 3B) and DEAE-cellulose (Fig. 2C and 3C) chromatograph

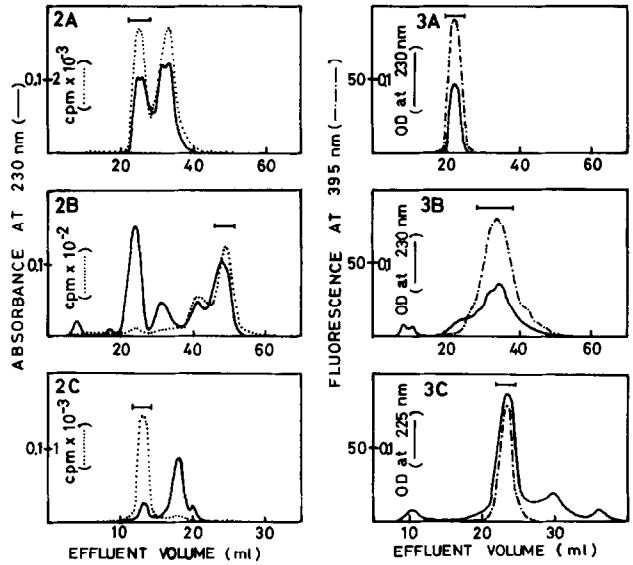

Fig. 2 and 3. Isolation of a pyridinoline-containing peptide (Fig.2) and a dihydroxylysinonorleucine-containing peptide (Fig.3). Details of the procedures are described in the text. (A) Sephadex G50 superfine rechromatography, (B) DEAE-cellulose chromatography, (C) phosphocellulose chromatography.

Table 1. Amino acid compositions of the isolated cross-link peptides and the proposed sequences[2].

Amino acid	76-90 + 990-23[c]	DHLNL-peptide	76-90 + (990-23[c])x2	Pyridinoline-peptide
4-Hyp	6	6(5.5)	10	10(9.6)
Asp	3	3(2.7)	6	6(5.6)
Thr	2	2(1.9)	3	3(3.2)
Ser	2	2(2.1)	4	4(4.3)
Glu	4	4(3.7)	8	8(7.9)
Pro	10	10(10.0)	20	20(20.3)
Gly	16	16(16.4)	27	27(27.4)
Ala	4	4(4.1)	7	7(7.2)
Val		(0.3)		(0.4)
Met	1	1(0.9)	1	1(0.7)
Ile		(0.2)		(0.4)
Leu	4	4(4.1)	6	6(6.0)
Tyr	1	1(0.9)	2	2(2.1)
Phe	1	1(1.0)	2	2(2.4)
Hyl	2	(0.1)	3	(0.2)
His	2	2(2.0)	3	3(2.8)
Lys		(0.1)		(0.2)
Arg	2	2(1.9)	3	3(3.0)
DHLNL[1]		1(0.8)		
Pyridinoline[1]				1(0.6)
Total	60	59	105	103

1) The molar color yields of DHLNL and pyridinoline were 1.35 (11) and 2.5 (7) times of that of leucine respectively.
2) Residuues per peptide. Values are expressed as round number. Actual values of average of the three analyses are shown in parentheses.

respectively. Final peptide preparations gave one band on SDS polyacrylamide gel electrophoresis.

The amino acid compositions of the final preparations of DHLNL-peptide and pyridinoline-peptide (peaks indicated by the bars in Fig. 2C and 3C) were shown in Table 1. They are so characteristic with their contents of histidine, arginine, serine, methionine and cross-link, that they provide enough informations for assigning the location in the fibrils from which the peptides derived, on the examinations of the available sequence data (2) and quarter stagger model collagen fibrils.

DISCUSSION

Dentin collagen has been often referred to as a suitable material for the study of collagen cross-linking because of its insolubility, homogeneity (type I collagen) and the simple profile of reducible cross-links (13,14). But the fact that there was no specific method of solubilization except CNBr treatment has rendered the study rather difficult (9). A simple maneuver of heating at 60°C after trypsin digestion dramatically solubilized the entity of dentin collagen (more than 75% of dentin collagen was solubilized only after this step). This technic could be also applied to bone, tendon and skin collagen, but, without this additional heating, no more than 50-80% (w/w) of these collagens could not be solubilized with trypsin (Kuboki et al., manuscript in preparation).

Amino acid composition of the purified DHLNL-peptide suggests that the peptide is double-chained with both C-termini of arginine, and one chain originates from the 45 residue sequence of 990 to 23^{C} in α1-CB6 and the other chain originates from 15 residue sequence of 76 to 90 in α1-CB4-5. The 59 residue cross-linked peptide with almost the same composition has been already isolated from bovine bone collagen and their counterpart peptides were identified as the sequence of 990 to 23^{C} and 76 to 90 in α1 chain, after the periodate cleavage of the cross-link (10). As discussed in the previous report (10), no other location for the peptide with this composition can be

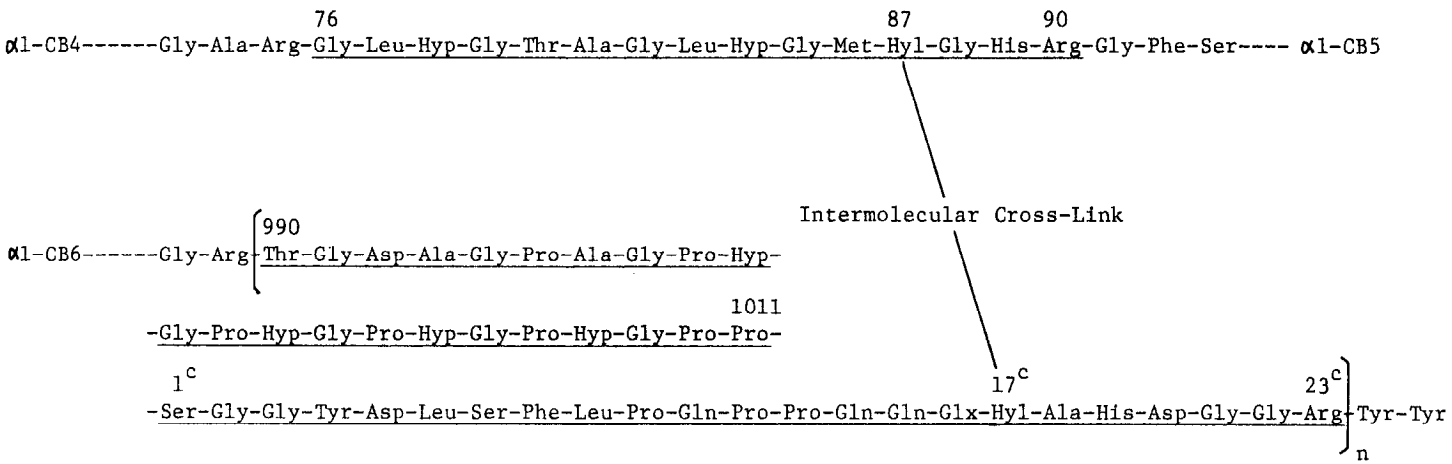

Figure 4. Amino acid sequences of the part of α1-CB4, α1-CB5 and α1-CB6 showing that the quarter stagger array of the two different chains coincides to form the amino acid compositions of the isolated cross-link peptides.

assigned in the quarter stagger model of the type I collagen molecules in the fibrils. The location of cross-link between the 87 and 17^{C} in α1 chain has been proposed by several authors in bone, tendon and skin collagens (1,3,8, 10). But this is the first report to identify the location in dentin collagen.

The amino acid composition of the pyridinoline peptide suggests that the peptide is triple-chained with their C-termini of arginine, and two chains originate from the sequence 990 to 23^{C} in α1-CB6 and one chain originates from the 76 to 90 in α1-CB4-5. Pyridinoline may arise from three hydroxylysine residues and the observed composition agrees with the calculation from the proposed sequences, except that the pyridinoline content was 0.6 residue instead of one per peptide. This might be ascribed to the fact that the pyridinoline becomes labile to acid hydrolysis, after it is processed at the high pH and temperature, or on ultraviolet irradiation (Dr. D. Fujimoto, personal communication). The location of the pyridinoline involving 87 and 17^{C} in α1 chain has been already assigned in bovine tendon collagen by isolating and identifying a pyridinoline containing peptide from the thermolysin digest of this collagen (7). From above data, the location of the two isolated peptides in collagen fibrils were proposed as Fig. 4.

Eyre and Oguchi (4) suggested that two residues of the keto-type DHLNL may convert to form one residue of pyridinoline. This idea was supported by the fact that incubation of dentin collagen at 37°C increased the content of pyridinoline at the expence of DHLNL content in this collagen (5). The evidences in the present paper that the major parts of DHLNL and pyridinoline in dentin collagen locate in the same position, strongly support above hypothesis. We suggest that the technics described in this paper will provide a useful tool to study the distribution of cross-links in collagen.

REFERENCES

1. Becker, U., Furthmayr, H.and Timpl, R. (1975) Hoppe-Syler's Z. Physiol. Chem. 356, 21-32
2. Bornstein, P. and Traub, W. (1979) The Protein IV, 411-632

3. Eyre. A.J. and Glimcher, M.L. (1973) Biochem Biophys. Res. Commun. 52, 663-671
4. Eyre, D.R. and Oguchi, H. (1979) Biochem. Biophys. Res. Commun. 92, 403-410
5. Eyre, D.R. Presented at 1st Int. Conf. Chem. Biol. Mineral. Tissue May, 1981, Chicago
6. Fujimoto, D., Akiba, K. and Nakamura, N. (1977) Biochem. Biophys. Res. Commun. 76, 1124-1129
7. Fujimoto, D. (1980) Biochem. Biophys. Res. Commun. 93, 948-953
8. Henkel, W., Rauterbert, J. and Stirtz, T. (1976) Eur. J. Biochem. 69, 223-231
9. Kuboki, Y., Tanzer, M.L. and Mechanic, G.L. (1973) Arch. Biochem. Biophys. 158, 106-115
10. Kuboki, T., Takagi, T., Shimokawa, H., Oguchi, H., Sasaki, S. and Mechanic G.L. (1981) Connect.Tissue Res. in press.
11. Kuboki, Y., Fujisawa, R., Aoyama, K. and Sasaki, S. (1979) J. Dent. Res. 58, 1926-1932
12. Kuboki, Y., Takagi, T. Sasaki, S., Saito, S. and Mechanic, G.L.(1981) J. Dent. Res. 60, 159-163
13. Mechanic, G.L.,Gallop, P.M. and Tanzer, M.L. (1971) Biochem. Biophys. Res. Commun. 45, 644-653
14. Scott, P.G. (1980) Biochemistry 19, 6118-6124