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TWO FLUORESCENT CROSSLINKING AMINO ACIDS HAVING N-SUBSTITUTED DIHYDROOXOPYRIDINE SKELETON ISOLATED FROM BOVINE ELASTIN

Kyozo Suyama* and Fumihiko Nakamura
Laboratory of Molecular Technology of Animal Products, Faculty of Agriculture, Tohoku University, Sendai 981, Japan

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Abstract: Two new fluorescent crosslinking amino acids named oxodesmosine and isooxodesmosine were isolated from bovine aorta elastin. These amino acids have unique structure, both N-substituted dihydrooxopyridine skeletons.

Elastin is one of the connective tissue proteins found in virtually every tissue and organs of mature animals and is major constituent of blood vessels and certain ligaments, and other compliant tissues such as lung and skin. The degradation of elastin is accelerated in several diseases such as atherosclerosis, emphysema and psedoxanthoma elasticum[1,2], and aberrations in elastin structure and metabolism have been detected in a number of heritable and acquired diseases[3].

Elastin is virtually insoluble, but is secreted from the cell as a soluble precursor, tropoelastin. As the tropoelastin come into contact with the extracellular matrix, lysine residues react post-translationally with lysyl oxidase to form reactive aldehyde, allysine. It has shown that allysines react with lysine to form stable quaternary pyridinium crosslinks, desmosine[4], isodesmosine[4], neodesmosine[5], allodesmosine[6] and pentasine[7]. It is known that elastin is one of the most stable mammalian protein. Turnover studies have suggested that, once laid down in aorta, the elastin remains virtually intact throughout life. However, the properties of a matrix metalloprotease, elastase, capable of degrading elastin have been reported during last few years[8]. The enzyme is secreted by granulocytes and macrophages. While deterioration of elastin fiber via degradation of crosslinks is not known.

We isolated two new fluorescent crosslinking amino acids named oxodesmosine (OXD) and isooxodesmosine (IOXD) from bovine aorta elastin. It

might be deduced from the structure of N-substituted dihydrooxopyridine skeleton that these new amino acids are metabolic intermediate of major pyridinium crosslinks of elastin, desmosine and isodesmosine, respectively, as shown in scheme.

As treatment of connective tissue with hot alkali, which is a conventional method to prepare elastin[9], destroyed the alkali-unstable oxopyridine crosslinks, bovine aortas which were obtained from fresh carcass of adult cows were cut into small segments, washed with saline, delipidated with chloroform/methanol (2:1, v/v) and then hydrolyzed in 6N HCl in conventional manner, at 110 ℃ for 48h. To prepare the unknown amino acids, 100g of an acid hydrolysate was charged on a large scale charcoal column (105 x 200 mm). Major lysine derived crosslinks were fractionated from water followed by elution with 20% aqueous methanol solution. Fraction containing lysine derived crosslinking amino acids was charged on preparative HPLC silica gel column(10 x 240 mm; Lobar LiChroprep Si60, Merck) using ethyl acetate/acetic acid/water, 2:1:1 v/v as a solvent. Individual fractions containing OXD and IOXD eluting on HPLC were pooled and evaporated to syrups, then a syrup was charged on a preparative HPLC ODS column (LiChroprep, 10 x 240 mm; Lobar, Merck) using a solvent of 0.02N HCl. Further purification of each crude OXD and IOXD fractions were done by same preparative column but using a solvent system of 0.1M phosphate buffer/acetonitrile, 5:1, v/v containing 20mM SDS (final pH4.0). Both purity of OXD and IOXD were confirmed by HPLC and TLC analysis. HPLC analysis were done by a Shimadzu LC-6A pump and SPD-6AV UV detector attached to a Superspher 100 RP-18 125-4 (Merck) reverse phase column using a solvent system of 0.1M phosphate buffer/acetonitrile, 5:1, v/v containing 20mM SDS (final pH4.0).

On elution systems of HPLC that resolve desmosine and isodesmosine, OXD and IOXD were eluted just before and just after desmosine, respectively and both fractions gave single peaks as shown in Figure 1. Both amino acids, OXD and IOXD, gave a heavy ninhydrine single spot on silica gel TLC using a solvent system of ethyl acetate/acetic acid/water, 2:1:1, v/v (both Rf: 0.08; both desmosine and isodesmosine were origin).

Both OXD and IOXD were very hygroscopic, white solid with a faint yellow tinges, soluble in aqueous solvents but not dry methanol, attempts to induce crystallization were unsuccessful.

The UV spectra were recorded on a Shimadzu UV-2100S spectrophotometer

and were shown on Figure 2. OXD exhibited absorption maximum at 310 nm in 0.1N HCl. The absorption maximum was not shifted in 0.1N NaOH. The absorption spectrum OXD was characteristic of N-substituted 1,2-dihydro-2-oxopyridine such as 1,2-dihydro-1-methyl-2-oxopyridine. IOXD exhibited absorption maximum at 263 nm in 0.1N HCl. The absorption maximum was irreversibly shifted gradually within 2h in 0.1N NaOH to 286 nm and then was reversibly shifted to 301 nm in 0.1N HCl. The absorption spectrum of IOXD was characteristic of N-substituted 1,4-dihydro-4-oxopyridine such as 1,4-dihydro-1-methyl-4-oxopyridine. The excitation and fluorescence maxima of

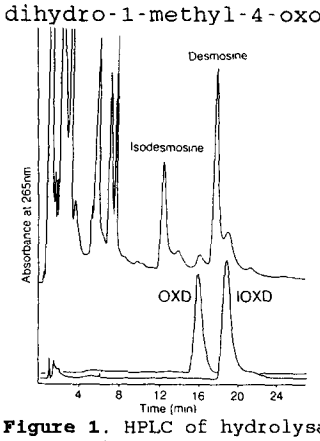


Figure 1. HPLC of hydrolysate of elastin, OXD and IOXD.

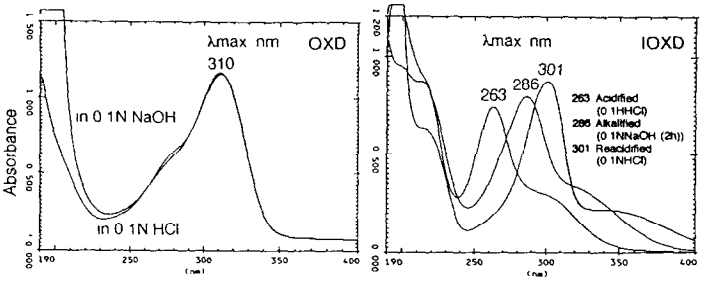


Figure 2. UV spectra of OXD and IOXD.

OXO were 334 and 400 nm, and that of IOXD were 365 and 424 nm in 0.1N HCl, respectively. FAB mass spectra of both OXO and IOXD, which were recorded on JEOL HX-105 instrument, gave same molecular weights of 495 (consistent with an elemental composition of both $C_{23}H_{37}N_5O_7$). ^{1}H-NMR and ^{13}C-NMR spectra of OXO and IOXD which were performed on JEOL JNM GSX-400 instrument were summarized as follows.

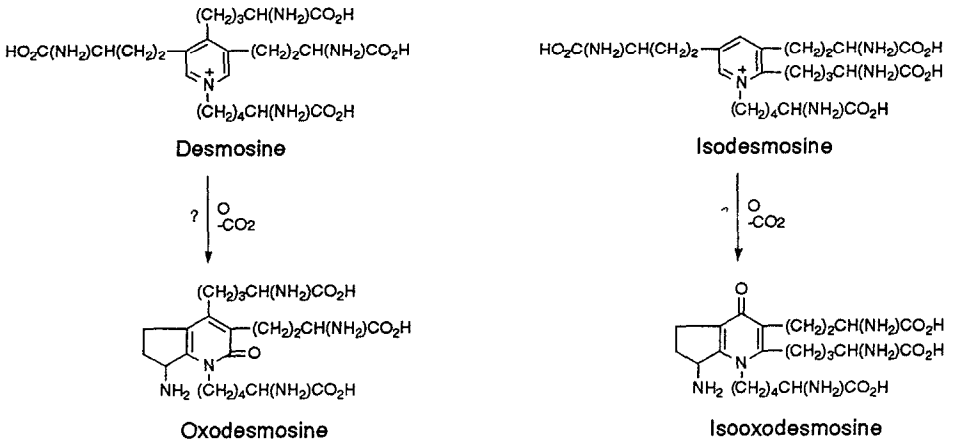
Oxodesmosine: NMR δ_H(400 MHz) 4.1-4.3 (2H, m, -C**H**$_2$-N<), 4.0-4.2 (3H, m, 3 x -C**H**(ND$_2$)COOD), 4.2 (1H, t, >C**H**-NH$_2$), 2.4-2.9 (6H, m, 3 x N-substituted 2-oxopyr-C**H**$_2$-); δ_C(400 MHz) 182.9 (>C=O), 174.9 and 174.4 (3 x -COOD), 144.8, 141.8, 125.8 and 122.4 (N-substituted 2-oxopyridine), 57.8 (>CH-NH$_2$), 56.5, 55.6 and 55.3 (3 x -CH(ND$_2$)COOD), 47.6 (-CH$_2$-N<).

Isooxodesmosine: NMR δ_H(400 MHz) 4.1 (1H, t, >C**H**-NH$_2$), 4.0 (3H, m, 3 x -C**H**(ND$_2$)COOD), 3.8 (2H, t, -C**H**$_2$-N<), 2.6-3.0 (6H, m, 3 x N-substituted 4-oxopyr-C**H**$_2$-); δ_C(400 MHz) 191.4 (>C=O), 174.7, 174.6 and 174.5 (3 x -COOD), 148.8, 135.6, 119.4 and 117.7 (N-substituted 4-oxopyridine), 57.2 (>CH-NH$_2$), 55.4 and 55.0 (3 x -CH(ND$_2$)COOD), 46.4 (-CH$_2$-N<).

From the spectrum data, we proposed the reasonable structures of OXO

and IOXD as shown in the scheme. It was found that both pure desmosine and isodesmosine which were obtained from bovine aorta elastin hydrolysate were not changed by the acid treatment used in the hydrolysis. The *in vivo* synthesis of OXO and IOXD were probably took place by oxidation of desmosine and isodesmosine as intermediate for further decompositions of such crosslinks. Although the mechanical details are not clear, one must invoke the cyclization, accompany with decarboxylation reaction during acid hydrolysis to form cyclopentene skeleton as shown in the scheme.

Scheme



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