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ISOTRITYROSINE, A NEW CROSSLINKING AMINO ACID ISOLATED FROM ASCARIS CUTICLE COLLAGEN

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Summary: A new amino acid was isolated from the cuticle collagen of Ascaris lumbricoides and characterized by ultraviolet, mass and nmr spectroscopies and chemical degradation. The results indicate that the compound is an isomer of trityrosine, having an ether linkage. The name "isotrityrosine" is proposed. Its structure suggests that it serves as a crosslink and plays a role in the organization of the collagen structure.

Collagen isolated from the cuticle of Ascaris lumbricoides has received attention from many investigators, since the collagen differs from collagens of other sources in several important respects (for review, see ref.1). The Ascaris collagen consists of subunits with a molecular weight of about 52,000 instead of the 300,000 molecular weight of tropocollagen from vertebrates (2,3). When the subunits were denatured, they lost the triple helical structure but retained their molecular weight (2). Thus, it is believed that a single polypeptide chain forms the triple-helical structure by self-folding rather than by association of three separate chains as occurs in vertebrate collagens (2). However, an alternative possibility that three polypeptide chains are held together by some as yet unidentified cross-linkages cannot be excluded (2,3).

We will report here the isolation and characterization of a new amino acid from Ascaris cuticle collagen. Its structure indicates that it serves as a cross-linkage and plays a role in the organization of the collagen molecules.

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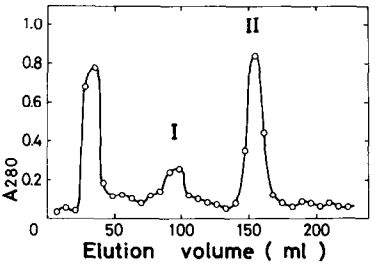


Fig.1 P-cellulose chromatography of the hydrolysate of Ascaris cuticle collagen. Details of the procedure are described in the text.

Materials and Methods

Ascaris lumbricoides were obtained from hog intestines at a local slaughter house. The cuticles were peeled off, cut into small pieces and washed with a large volume of 0.5M NaCl at 4°C for 24 hr. Reduced and carboxymethylated collagen was prepared according to McBride and Harrington (2) and used in earlier experiments. In later experiments, collagen was extracted from the cuticle with 0.1M sodium phosphate buffer, pH 7.0, containing 1% 2-mercaptoethanol (4) and then dialyzed and lyophilized. The same results were obtained with both preparations.

Collagen (0.5 g) was hydrolyzed in 6N HCl (20 ml) at 110°C for 24 hr in a sealed tube. The solution was evaporated under vacuum and the hydrolysate was taken up in water and applied on a P-cellulose column (H^+ form, 1.8 x 15 cm). Elution was performed with a linear gradient formed from 150 ml of water and 150 ml of 0.5N HCl. Ultraviolet absorbing peaks (peak II in Fig.1) obtained in several separate runs were pooled, evaporated under vacuum, taken up in water, and applied on a small column of P-cellulose (H^+ form). After washing with water, the compound was eluted with 2N NH_4OH. The eluate was dried under vacuum and the residue was taken up in water and applied on a DEAE-cellulose column (1.8 x 15 cm) which had been equilibrated with 5 mM Na_2HPO_4. The compound was then eluted with 20mM Na_2HPO_4, and desalted by adsorption on a P-cellulose column and elution with 2N NH_4OH. The compound was taken up in a minimum amount of water and precipitated with ethanol. About 20 mg was obtained from 10 g of collagen.

Dityrosine and trityrosine were prepared and separated according to Andersen (5).

The ^1H- and ^{13}C-nmr spectra were measured in D_2O on a Nicolet NT-360 spectrometer and JEOL JNM-FX-100 spectrometer, respectively, and all chemical shifts were reported in parts per million (δ) downfield from TSP [sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl) propionate] as external standard. Field desorption mass spectrum was obtained on a JEOL JMS-01SG-2 mass spectrometer.

Results and Discussion

The typical chromatographic pattern of the hydrolysate of Ascaris cuticle collagen is shown in Fig.1. Peak I and II

Table 1. Properties of tyrosine, dityrosine, trityrosine and the unknown amino acid.

Amnino acid	Ultraviolet absorption maximum (nm)		Paper chromatography (Rf)			
	acid	alkaline	A	B	C	D
Unkown amino acid	280	300	0.18	0.01	0.07	0.40
Tyrosine	275	293	0.50	0.28	0.57	0.54
Dityrosine	283	317	0.25	0.01	0.18	0.36
Trityrosine	286	322	0.16	0.00	0.03	0.30

Solvent A: n-butanol-acetic acid-water (4:1:2), B: isopropanol-conc. ammonia-water (80:10:10), C: t-butanol-formic acid-water (75:15:15), D: phenol-water (4:1).

corresponded to the elution positions of dityrosine (I in Fig.4) and trityrosine (II in Fig.4), respectively. However, the material in Peak II differed from trityrosine in that it was eluted from DEAE-cellulose with 20 mM Na_2HPO_4 whereas trityrosine was not eluted under the conditions (6).

The compound purified from Peak II gave a single ninhydrin positive spot on paper chromatograms and showed a blue fluorescence in ultraviolet light after the paper was exposed to ammonia vapour (6). It gave a blue color with Folin's reagent for phenols (6). The ultraviolet absorption maximum is at 280 nm in acid solution and at 300 nm in alkaline solution. As summarized in Table 1, the compound resembles dityrosine and trityrosine, indicating that it is an analogue of tyrosine oligomers.

Field desorption mass spectrum exhibited an intense peak at m/e 540, which corresponded to $[M+1]^+$ ion peak for an oxidatively coupled trimer of tyrosine. The 360 MHz ^{1}H-nmr spectrum (Fig.2A) also indicated the trimeric structure. It showed three sets of ABX pattern for $-C\underline{H}_2C\underline{H}(\overset{+}{N}H_3)COO^-$ [A:2.81(1H, dd,J=14,8); 2.90(1H, dd, J=14,8); 2.99(1H, dd, J=14,8), B:2.96(1H, dd, J=14,5); 3.06 (1H, dd, J=14,5); 3.15(1H, dd, J=14,5), X:3.71(1H, dd, J=8,5); 3.77(1H, dd, J=8,5); 3.84(1H, dd, J=8,5 Hz)] and the signals for nine aromatic protons [6.70(1H, s), 6.75-6.82(4H, m), 7.02(1H,d),

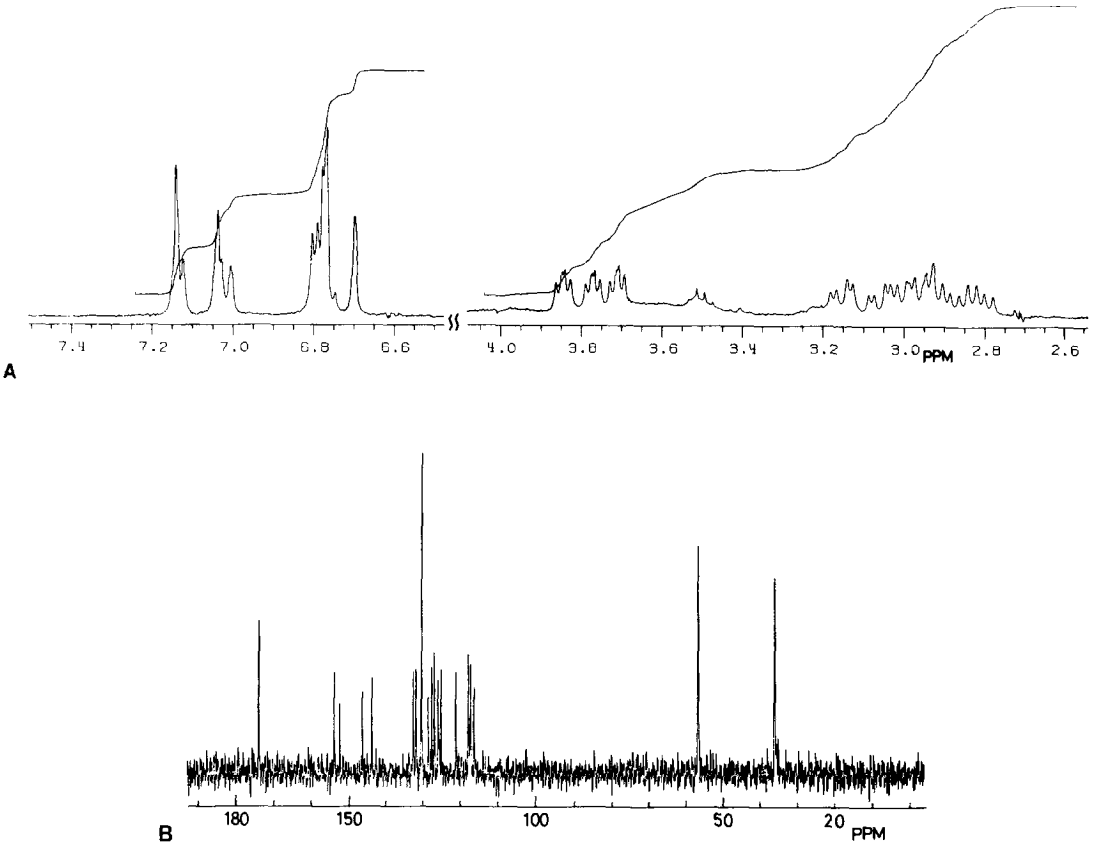


Fig.2 ^{1}H-nmr spectrum (A) and proton-decoupled ^{13}C-nmr spectrum (B).

7.04(1H, s), 7.13(1H, d), 7.14(1H, s)], one more than those of trityrosine. Twenty signals in the ^{13}C-nmr spectrum (25.0 MHz, Fig.2B) appeared at 37.53(t), 58.09(d), 118.26(d), 119.20(d), 119.84(d), 123.18(d), 127.16(s), 127.97(d), 129.00(s), 129.64(s), 130.61(s), 132.45(d), 132.45(s), 133.94(d), 134.70(d), 145.79(s), 148.33(s), 154.39(s), 155.85(s) and 175.89(s). The four signals of oxygen-substituted aromatic ring carbons at 145.79, 148.33, 154.39 and 155.85 in ^{13}C-nmr spectrum and the number of the aromatic proton signals in ^{1}H-nmr spectrum indicate that the three tyrosine units are combined by one biphenyl and one ether linkages in the molecule, instead of the two biphenyl ones.

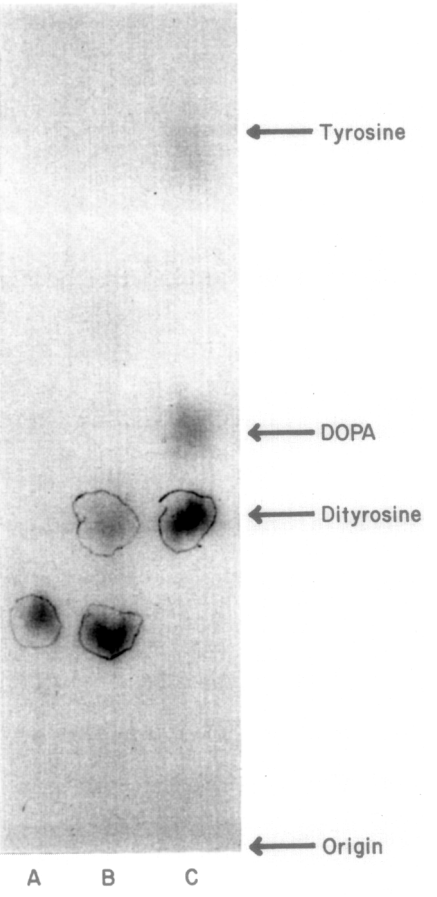
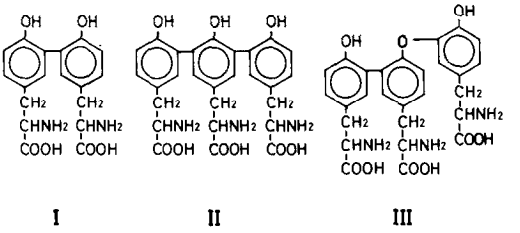


Fig.3 Paper chromatogram of alkaline hydrolysates. The isolated amino acid (ca.0.5 mg) was heated in 4N NaOH (0.5 ml) at 110°C for 24 hr in a sealed tube. The solution was then neutralized, desalted and a portion was applied on Toyo No.51 filter paper and developed in n-butanol-acetic acid-water (4:1:2). Visibly fluorescent spots were marked with a pencil, and then amino acids were located with ninhydrin. A: untreated amino acid, B: alkaline hydrolysate, C: dityrosine, DOPA and tyrosine.

As phenylether is normally subject to alkaline hydrolysis, the compound was heated in 4N NaOH at 110°C for 24 hr. A portion was hydrolyzed, yielding ninhydrin positive products (Fig.3). The major product was identified as dityrosine by paper chromatographic behaviors and ^{1}H-nmr spectra (data not shown). Therefore, one tyrosine is linked to the hydroxyl group of dityrosine by ether linkage. The site of the linkage on the tyrosine should be



I II III

Fig.4 Structures of dityrosine (I) and trityrosine (II), and the proposed structure for the amino acid isolated from Ascaris cuticle collagen (III).

6-position, considering the chemical and enzymic phenolic oxidation mechanisms (7). On the other hand, the formation of DOPA (3,4-dihydroxyphenylalanine) is expected in alkaline hydrolysis, however, it is too labile to detect after the hydrolysis.

From these data, the most probable structure for the compound is proposed as shown in Fig.4 - III. As far as we know, the occurrence of this compound in proteins has not been demonstrated. We propose the trivial name "isotrityrosine" for this compound.

The compound may be synthesized from three tyrosine residues in polypeptide chains by the action of a peroxidase (7). It is of interest that the cuticle collagen contains isotrityrosine but contains only a trace amount of dityrosine and virtually no trityrosine, whereas cuticlin, another component of the cuticle(4), contains appreciable amounts of dityrosine and trityrosine (8) but does not contain isotrityrosine. A special conformation or packing of peptide chains may be necessary for the formation of this amino acid. This fact also indicates that isotrityrosine was not formed artifactually during the acid hydrolysis of the protein.

The structure of isotrityrosine suggests that it links three polypeptide chains and play a role in the organization of the

molecular structure of Ascaris cuticle collagen. Although the exact content of isotrityrosine has not been determined, the yield of the amino acid indicated that the content is 0.6 residue/ 1000 total residues or more. It has been considered that Ascaris cuticle collagen has an unusual structure that a single polypeptide chain forms the triple-helix by self-folding rather than by association of three separate chains (2). However, the presence of isotrityrosine in this collagen suggests that such unusual structure might not be necessarily considered.

Acknowledgements

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