EVIDENCE FOR A PREVIOUSLY UNDETECTED SEQUENCE AT THE CARBOXYTERMINUS OF THE α1 CHAIN OF CHICKEN BONE COLLAGEN

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SUMMARY

The peptides tyrosyltyrosine and Asp,$(Gly)_2$,Tyr,Arg have been isolated respectively from tryptic digests and chymotryptic digests of chicken bone collagen solubilized in denaturing solvents. By homology to the structure of calf skin collagen, the peptides appear to derive from the carboxyterminus of the α1 chain. To confirm this, α1 and α2 chains were isolated from bone gelatin and tyrosyltyrosine was recovered from a tryptic digest of α1 but not from α2. Since tyrosine is not present at the carboxyterminus of the α1 chain of neutral salt-soluble and acid-soluble bone collagen of lathyritic chicks, it is concluded that a carboxyterminal sequence is missing from α1 chains of such extracts.

The relative sequence and composition of all the CNBr-peptides of α1 and α2 chains of chicken bone (1,2,3) and skin (4,5,6) collagens has been reported. The data suggest that collagen in the two tissues has the same primary structure (5). Tyrosine is apparently restricted to two residues in the amino terminal region of α1, and one residue near each end of α2. Recently, peptides containing tyrosine have been isolated from the carboxyterminus of α1 chains of calf skin collagen (7,8,9). Intact α1 chains bearing the complete carboxy-terminus were found only in collagen extracted in 8M urea. Chains extracted in neutral salt or citrate buffer lacked either a single tyrosine residue, or a sequence of sixteen amino acids including the terminal -tyr-tyr-COOH and a lysine that was usually converted to an aldehyde in intact chains, presumably for participation in crosslinking.

This report describes the isolation from chicken bone collagen of tyrosyl peptides that cannot be accomodated in the known structures of α1 and α2 chains.

On the basis of their composition and manner of isolation in comparison to the peptides of calf skin collagen (9), it is concluded that they derive from a previously undetected sequence at the carboxyterminus of α1 chains of chicken bone collagen.

MATERIALS AND METHODS

Preparation of Bone Collagen. The diaphyseal regions from metatarsal bones of 12-14 week old chickens were thoroughly cleaned, dried, and then powdered in a micro-mill cooled by a mixture of methyl cellusolve and dry ice. The bone powder was demineralized in 0.2M EDTA, pH 7.9, at 4°C for 6 days, then thoroughly washed in water and freeze-dried. A large proportion (20-40%) of the collagen was solubilized by stirring the powdered bone matrix in the denaturing solvents 9M LiCl, 5M KCNS or 4M $CaCl_2$ for 16 hours at room temperature (24°C) or 1 week at 4°C (10). The supernatant and residue were dialyzed exhaustively against distilled water, then freeze-dried. Component α1 and α2 chains were isolated by chromatography of the gelatin on CM-cellulose (11), pooling only the latter half of the α2 peak to avoid contamination with β_{12}.

Digestion with Trypsin and Chymotrypsin. Samples (50-100 mg) of bone collagen were digested with trypsin (Worthington, TPCK-treated, 2 x cryst.) as described (9). However, a higher ratio of enzyme to substrate was used (1 to 10 by weight). For chymotryptic digestion, bone collagen was stirred in 0.1M NH_4HCO_3, pH 7.8, (10-20 mg collagen/ml) at 15°C for 24 hours with α-chymotrypsin (Worthington, 3 x cryst.), at an enzyme to substrate ratio of 1 to 100 by weight.

Isolation of Tyrosyltyrosine. Freeze-dried tryptic digests were redissolved in 5 ml of 0.1M acetic acid and eluted from a column of Bio-Gel P-2 (200-400 mesh). The tyrosyltyrosine was resolved as a retarded peak emerging after salt (*Figure 1*).

Isolation of Chymotryptic Peptides. Diffusible peptides were recovered quantitatively by ultrafiltration of the chymotryptic digest through dialysis membrane. The freeze-dried diffusate was chromatographed on a column of phosphocellulose at 40°C (*Figure 2*). Peptide peaks of interest in the

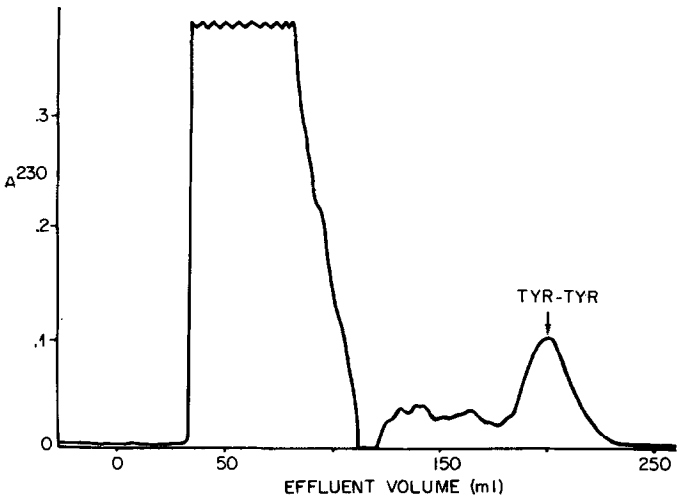

Figure 1 Elution of a tryptic digest of KCNS-extracted chicken bone collagen from a column (2.5 x 30 cm) of Bio-Gel P-2, 200-400 mesh in 0.1 M acetic acid. Flow rate was about 100 ml/hr.

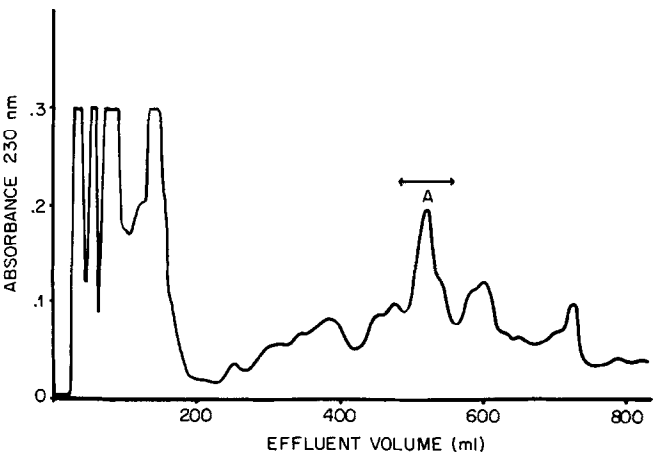

Figure 2 Column chromatography on phosphocellulose (Whatman P11, 2 x 20 cm) at 40°C of diffusible peptides obtained by ultrafiltration of a chymotryptic digest of LiCl-insoluble chicken bone collagen. A linea gradient of 0-0.3 M NaCl in 800 ml of 0.001 M sodium acetate, pH 3.6, was applied at a flow rate of 150 ml/hr.

phosphocellulose effluent were further resolved by chromatography on Bio-Gel P-4 (*Figure 3*) or P-2.

Peptides were hydrolyzed in 6N HCl for 24 hours at 105°C, and $^{M}/_{100}$ phenol was included in the hydrolysate to prevent destruction of tyrosine. Amino acid analyses were performed on a single-column automated analyzer.

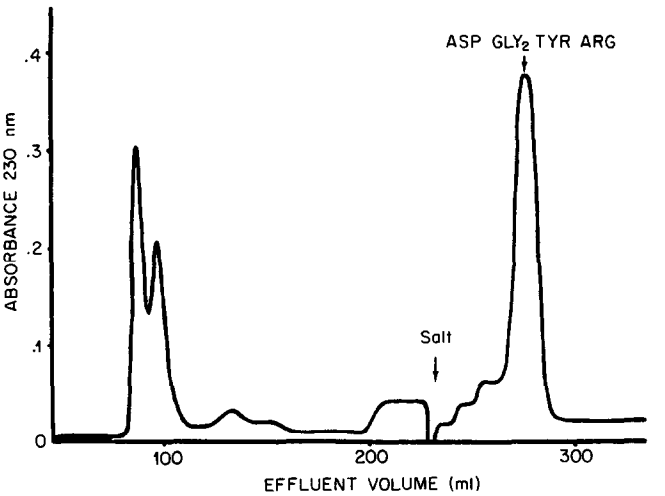

Figure 3 Elution of material in peak A of the phosphocellulose chromatogram (*Figure 2*) from a column of Bio-Gel P-4, 200-400 mesh (1.5 x 150 cm) in 0.1 M NH_4HCO_3, pH 7.8, at room temperature. Flow rate was 19 ml/hr.

RESULTS

The peptide Tyr-Tyr was identified by the recovery of tyrosine as the only amino acid after acid hydrolysis. Also, when applied to the analyzer without hydrolysis, the only ninhydrin-positive peak, apart from ammonia, was a broad peak eluting after histidine. The color yield of the broad peak was exactly half that of the tyrosine from an equal amount of hydrolyzed material, as might be expected for Tyr-Tyr. Similar amounts of Tyr-Tyr, 0.4 ± .05μM, were recovered from 100 mg samples of the three gelatin extracts (KCNS, LiCl and $CaCl_2$) and the insoluble residues. Assuming the ratio of α1 to α2 chains in the collagen is 2 to 1, then the amount of tyr-tyr recovered could be accomodated at the carboxytermini of about two-thirds of the α1 chains. Approximately 0.2μM Tyr-Tyr was recovered from a tryptic digest of 30 mg of the α1 fraction, but less than 0.01μM Tyr-Tyr was recovered from a similar weight of α2.

The peptide Asp,Gly_2,Tyr,Arg eluted in peak A and was discovered after analysis of several of the peaks eluting from the phosphocellulose column (*Figure 2*). Similar amounts of other tyrosyl peptides eluted near the beginning

TABLE I

Amino Acid Composition of the Tyrosyl Peptide Isolated from Chymotryptic Digest of LiCl-Extracted and LiCl-Insoluble Chicken Bone Collagen

	Res/Molecule	
	LiCl-Extracted*	LiCl-Insoluble†
ASP	1 (0.94)	1.0
GLY	2 (2.00)	2.4
TYR	1 (1.00)	1.0
PHE	0 (0.00)	0.4
ARG	1 (1.09)	1.4

* Isolated by column chromatography on Bio-Gel P-4. Other amino acids were not detected above .005 res/molecule.

† This peptide preparation was isolated by chromatography on Bio-Gel P-2 and was apparently contaminated with the tripeptide Gly,Phe,Arg. About equal amounts of the two peptides were recovered after elution of peak A (*Figure 2*) from a 2.5 x 30 cm column of Bio-Gel P-2, 200-400 mesh. The contaminating Gly,Phe,Arg was partially removed by re-chromatography on an 1.3 x 100 cm Bio-Gel P-2 column.

of the chromatogram and had the compositions Glu,Met,Ser,Tyr; Ser,Tyr and Glu,Tyr that might be expected from chymotryptic cleavage of the known amino-terminal sequences of α1 and α2 chains (12). The unexpected tyrosyl peptide was recovered from chymotryptic digests of a 9M LiCl extract and the 9M LiCl-insoluble residue of chicken bone collagen. Data on its amino acid composition are given in *Table I*. About 0.2μM peptide were recovered from 100 mg of either extracted or insoluble collagen.

DISCUSSION

The carboxyterminal sequence in α1 chains of calf skin collagen extracted in 8M urea is -Ala-His-Asp-Gly-Gly-Arg-Tyr-Tyr (9). The recovery of a relatively large amount of Tyr-Tyr from a tryptic digest of the α1 chains of chicken bone collagen indicates that a similar carboxyterminal region is present in the α1 chain of this collagen. The peptide Asp,$(Gly)_2$,Tyr,Arg, isolated from a chymotryptic digest of chicken bone collagen, probably derives from the same carboxyterminal sequence. Previously, this peptide was recovered from the above

sequence in α1 of calf skin collagen after chymotryptic digestion (9).

Acid-soluble collagen from lathyritic chick bone, and neutral salt-soluble and acid-soluble collagen from normal and lathyritic chick skin, apparently have the same primary structure (1-6). The peptide Tyr-Tyr cannot be accomodated in either the α1 or the α2 chain of this collagen. It is presumed that, similar to the finding with calf skin collagen (9), an extra-helical sequence is lost from α1 when bone collagen - and probably skin collagen also - is extracted in neutral salt and dilute acid from tissues of either normal or lathyritic chicks. The sequence may be lost by the action of proteolytic enzymes (9), or perhaps by rupture of a peptide bond which is unusually labile in acid. A loss of terminal regions from a proportion of α1 chains could explain a previous finding that two types of α1 chain, one with a slightly higher molecular weight, can be resolved from an extract of chicken bone collagen (13,14).

The recovery of a similar amount of Tyr-Tyr from bone collagen solubilized by denaturation in 4M $CaCl_2$, 9M LiCl or 5M KCNS, and from the insoluble collagenous residues, indicates that the collagen chains extracted by these reagents are more representative of native, intact chains than are neutral salt-soluble and acid-soluble collagen from either normal or lathyritic tissues. Although it has been suggested that the collagen extracted from bone by denaturing agents was extensively degraded (15), presumably by cleavage of peptide bonds (16), it was originally contended that the solubilization was mediated by disruption of relatively labile intramolecular and intermolecular bonds (10). Recent evidence supports this original contention, and indicates that labile aldimine crosslinkages, rather than peptide bonds, break when bone collagen is exposed to these denaturing solvents (17). The present findings support this concept.

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