DIFFERENCES BETWEEN CNBr PEPTIDES OF SOLUBLE AND INSOLUBLE BOVINE COLLAGENS*

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SUMMARY

The compositions of the cyanogen bromide peptides of insoluble bovine skin and dentin collagens are nearly identical with those obtained from the soluble collagen. However, some qualitative and quantitative differences are present. The insoluble collagens do not go completely in solution after the treatment. The insoluble residues have compositions showing a firm association of collagen with non-collagenous peptides. The content of peptide α1-CB6 is sharply reduced in both insoluble skin and dentin collagen, indicating involvement of this peptide in the formation of intermolecular cross-links. Several heterogeneities were noted regarding many peptides. In particular, two forms of α1-CB3 and of α1-CB6 were isolated in agreement with the idea that two types of α1-chains are present. The situation is more complex, in that the second form of α1-CB3 from skin and dentin are not identical.

After stabilization by borohydride reduction, a number of new amino acids have been identified in insoluble collagen (1,2,3), supporting the opinion that in their non-reduced form, these act as intermolecular cross-links. The precise location and isolation of the intermolecular cross-link containing collagen-peptides has not yet been accomplished. One way seemed to be to apply the technique of cyanogen bromide (CNBr) cleavage to tissues which are insoluble and supposedly rich in cross linkages. Having isolated and characterized the CNBr peptides from α1 and α2 chains of acid soluble bovine skin collagen (SC) (4), we have analyzed and compared the CNBr digestion of insoluble bovine skin collagen (IC) and dentin bovine collagen (DC), both supposedly containing covalent

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intermolecular cross-linkages.

METHODS. The preparation of the bovine skin collagen was as previously described (4). The IC fraction was the residue left after neutral salt extractions and 4 extractions with acetic acid. β_{12} was isolated from SC in the same CM-cellulose chromatographic separations used to obtain the pure α1 and α2 chains (4). Bovine dentin collagen was prepared from unerupted teeth following the procedure of Veis and Schlueter (5). The dentin was extracted repeatedly at 4°C with 0.5 M EDTA, adjusted to pH 7.4 until the calcium content was reduced below the limit of detection. This process took 11 extractions After that, dentin was washed with distilled water to remove all EDTA, lyophilized and stored.

Aliquots of IC, DC or β_{12} of SC, weighing 400 mg were suspended in 50 ml of 70% formic acid, flushed with nitrogen. A quantity of CNBr equal to twice the weight of the sample was added. The digestions were carried out at room temperature (24°C) for 4 hrs in the case of β_{12} and IC and 8 hrs for DC. At the conclusion of the reaction, the mixture was diluted and lyophilized.

The lyophilized material was dissolved in 0.15 M acetic acid and filtered through a sintered-glass filter of medium porosity. A small portion retained on the filter was collected and dried.

The filtrate was freed of residual salts by filtration on a 4 x 40 cm column of Bio-Gel P-2 (100-200 mesh), equilibrated with 0.15 M acetic acid. The fractions containing the peptides were combined and lyophilized.

The CNBr peptides were chromatographed on a 2.5 x 12 cm column of CM-cellulose, equilibrated at 41°C with 0.02 M sodium citrate, pH 3.6. The elution was carried out with a linear gradient of NaCl from 0 to 0.14 M over a volume of 2000 ml. Fractions comprising a single peak were combined, lyophilized and desalted through Bio-Gel P-2.

The components of the initial peptide peaks were purified further by rechromatography with CM-cellulose equilibrated with 0.02 M sodium acetate, pH 4.8, already described (4) or by gel filtration on Bio-Gel P-4 or Bio-

Gel P-30 equilibrated with 0.15 M acetic acid, or by agarose molecular sieve chromatography on a 2.5 x 150 cm column of Bio-Gel A-1.5 (200-400 mesh), equilibrated with 1 M $CaCl_2$ containing 0.05 M TRIS and adjusted to pH 7.5. Molecular weights of the isolated peptides were determined from their elution volumes on a calibrated agarose column identical to that described by Piez (6), using tritiated water (THO) as a standard for the total volume. Amino acid analyses were performed on an automatic amino acid analyzer (JEOLCO) after hydrolyses in constant boiling HCl at 108°C for 24 hrs under nitrogen in sealed tubes. Gel electrophoresis was done using the procedure described earlier (4). Phosphate was determined with the method of Lucena-Conde and Prat (7).

RESULTS AND DISCUSSION. Neither IC, after 4 hrs of treatment with CNBr, nor DC, after 8 hrs of the same treatment, went entirely into solution. To separate the supernatants, the digests were filtered through a medium porosity sintered-glass filter. In the case of IC, 85% of the starting material was filterable, determined by hydroxyproline measurement (8). In the case of DC, 92% of the starting material was recovered, but still some material remained on the filter.

To show that the breakdown was not made by the acidity of the medium, control experiments were carried out with insoluble collagen or dentin collagen reacting under the same conditions but without CNBr. A small amount of collagen went into solution (about 10% in the case of IC and 2% in the case of dentin). With gel electrophoresis and CM-cellulose chromatography, the material was shown to be α's and β's. No unspecific peptides were demonstrated and no peaks appeared in CM-cellulose using the same elution and gradients of buffers used for the separation of CNBr peptides.

The amino acid composition of SC, IC, CNBr extracted collagen, and CNBr residue collagen are shown in Table I.

The soluble and insoluble collagens are nearly identical in amino acid composition, but a clear difference is noticeable between the filtrate of CNBr-digest of IC and the CNBr residue of IC. This last material has an in-

TABLE I

Amino Acid Composition[a]
(residues/1000 total residues)

	SC	IC	CNBr extract from IC	CNBr residues from IC
Hydroxyproline	94	94	95	86
Aspartic Acid	46	46	45	51
Threonine	16	17	16	20
Serine	33	36	34	39
Proline	126	127	127	118
Glutamic Acid	73	72	72	82
Glycine	333	326	331	326
Alanine	114	112	112	108
Valine	20	23	22	30
Isoleucine	11	11	10	14
Leucine	24	25	24	30
Tyrosine	3	3	3	3
Phenylalanine	13	13	13	14
Hydroxylysine	6	6	6	3
Lysine	27	27	27	22
Histidine	4	5	5	3
Arginine	51	51	51	48
Methionine[b]	6	6	-	3
Homoserine[c]	-	-	6	-
Total	1000	1000	999	1000

[a] Residues rounded off to the nearest whole number
[b] Includes methionine sulfoxide
[c] Includes homoserine lactone

creased content of aspartic acid, glutamic acid, serine, threonine, isoleucine and leucine and a decreased content of hydroxyproline, proline and hydroxylysine. The high value of hydroxyproline shows that the insoluble material represents mainly collagen but it contains an additional moiety rich in the acidic amino acids.

Similar data are shown in Table II for bovine dentin collagen, CNBr extracted dentin and CNBr residue dentin collagen. One interesting difference between skin and dentin collagens is the much higher content of hydroxylysine in dentin collagen. The other amino acids do not vary very much.

The CNBr residue dentin collagen presents a very high content in aspartic acid, serine and tyrosine, an increase in threonine, isoleucine, leucine and a decrease in glycine, alanine, hydroxyproline and hydroxylysine. Moreover, while the IC and DC residues are similar in nature, the residue from DC con-

TABLE II

Amino Acid Composition[a]
(residues/1000 total residues)

	DC	CNBr extract from DC	CNBr residue from DC
4-Hydroxyproline	102	101	74
Aspartic Acid	46	46	85
Threonine	17	16	21
Serine	37	35	83
Proline	118	120	114
Glutamic Acid	72	73	74
Glycine	332	337	278
Alanine	109	105	83
Valine	20	20	22
Isoleucine	10	10	15
Leucine	25	25	35
Tyrosine	4	3	9
Phenylalanine	13	14	16
Hydroxylysine	10	10	7
Lysine	23	23	25
Histidine	5	5	6
Arginine	51	51	50
Methionine[b]	6	-	3
Homoserine[c]	-	6	-
Phosphate	(6.2)	(2.3)	(45)
Total	1000	1000	1000

[a] Residues rounded off to the nearest whole number
[b] Includes methionine sulfoxide
[c] Includes homoserine lactone

tains phosphate and has a much higher content of aspartic acid and serine than that from IC. The DC residue corresponds to collagen containing the bound phosphoprotein described by Veis and Perry (9).

The CNBr collagen peptides were subjected to CM-cellulose chromatography as is illustrated in Figure 1. The peaks are labeled by number corresponding to the sequence of elution of the peptides in IC and DC. The CNBr peptides from β_{12}, isolated from SC gave the same chromatogram as a mixture of α1 and α2-chains (4). The three chromatograms are similar, but there are some distinct differences. Peaks 3 and 4 of IC and DC are not present in SC. Peptide 7 is decreased in both insoluble collagens. In IC a small peak remained, while in dentin, the peak is reduced to a shoulder of peak 8. The region corresponding to peaks 9, 10, 11 is less well resolved in DC than in SC or IC.

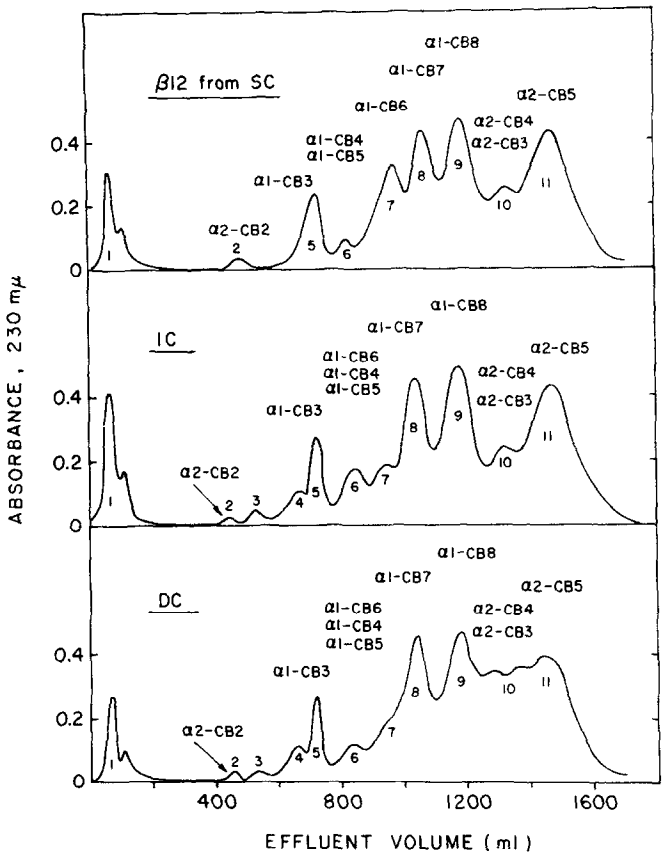

Fig. 1. CM-Cellulose chromatograms of the CNBr peptides from β_{12} of acid soluble bovine skin collagen (SC), insoluble skin collagen (IC) and dentin collagen (DC).
Elution was achieved with a linear gradient from 1000 ml of starting buffer (0.02 M sodium citrate, pH 3.6) and 1000 of limit buffer (starting buffer containing 0.14 N NaCl).

The major components were purified and isolated from every peak. In SC this was a relatively easy task, except for the purification of peptides from α2 chains. The major peptides all have some minor components with similar amino acid composition. In IC and DC, the purification was more difficult because of additional fractions which were present in every peak. Particularly during rechromatography in acetate buffer at pH 4.8, various fractions were shown which have about the same molecular weights on agarose columns, but slightly different amino acid compositions. The relative amounts of the apparently homologous peptides varied.

The isolated major components were identified by comparison with the amino acid composition of the peptides from SC. The compositions from SC and IC were very close. In DC the only exception was that the content of hydroxylysine was always increased in every lysine containing peptide. However, the sum (lysine + hydroxylysine) was the same in equivalent peptides in all three collagens.

The real difference between IC and DC was in the sharp reduction of α1-CB6 and the presence of a new peak (peak 4) (peak 3 was found to contain α2-CB2 and an uncleaved peptide α1-CB2-4). Peptide α1-CB6 was not only sharply reduced, but the remaining quantity was heterogeneous. A part of peptide α1-CB6 was found in peak 6 and another portion in peak 7, easily separated with Bio-Gel P-4 from α1-CB4 and α1-CB5, which are also contained in the same peaks.

Peptide 4 was purified and it was found that this fraction was different in IC and DC. The peptide has a molecular weight slightly smaller than α1-CB3.

TABLE III

Amino Acid Composition of α1-CB3 Peptide[a]

	Insoluble Collagen		Dentin Collagen	
	Peak 4	Peak 5	Peak 4	Peak 5
4-Hydroxyproline	14	14	14	15
Aspartic Acid	6.2	6.5	6.3	6.4
Threonine	2.3		2.1	
Serine	2.8	2.8	4.3	2.8
Glutamic Acid	12	16	11	15
Proline	19	16	18	15
Glycine	51	51	51	51
Alanine	15	22	14	23
Valine	2.8	3.8	3.6	3.9
Isoleucine	1.3		1.4	
Leucine	2.7	3.0	3.8	3.4
Tyrosine				
Phenylalanine	1.3	3.0	3.3	2.6
Hydroxylysine	0.4	0.3	1.4	1.4
Lysine	6.0	4.8	4.5	3.9
Histidine				
Arginine	4.2	6.0	6.4	6.1
Homoserine[b]	0.9	0.9	1.0	0.9
Total	141	150	145	150

[a] Actual values are listed for amino acids present as less than 10 residues. A space indicates less than 0.2 residues.
[b] Includes homoserine lactone.

The amino acid composition has some homology to α1-CB3 (Table III), but bigger differences are found in relation to threonine, glutamic acid, proline, alanine, phenylalanine.

Recently, Miller (10) found that human skin contains two α1-CB3 peptides, apparently derived from genetically distinct α1 (I) and α1 (III). The position in CM-cellulose chromatography of purified peptides from peak 4 and the variations in amino acid composition with α1-CB3 are of the same type as the ones found by Miller in human skin. Interestingly enough, the peptide purified from peak 4 in dentin collagen not only shows small differences from α1-CB3 from peak 5, but it is also different from the α1-CB3 from peak 4 of IC (Table III).

CONCLUSION. As expected, the compositions of the major CNBr peptide components of IC and DC are nearly identical with those obtained from the soluble collagen, although about 80% of the skin collagen and nearly all of the dentin collagen are insoluble. However, it is also evident that important tissue specific differences exist both qualitatively and quantitatively.

One major area of difference is in relation to the α1-CB6 peptides of both IC and DC. In these collagens, the content of α1-CB6 is sharply reduced. Using the present techniques, a cross-linking peptide has not been found. However, from the marked reduction in α1-CB6 it seems reasonable to postulate that α1-CB6 is involved in the intermolecular cross-linkage region. Although not detailed in this report, the remaining α1-CB6 is divided into two peaks of slightly different molecular weight and amino acid composition. The presence of the new α1-CB6 components can be explained in terms of the presence of covalent attachments to the -COOH-terminal region or to sequence heterogeneity in the peptides.

Several other heterogeneities have been noted in IC and DC, giving the impression of the existence of a different α1 chain in each type of collagen. In particular, two types of peptides related to α1-CB3 are clearly present. In both IC and DC, the α1-CB3 of peak 4 accounts for about 30% of the total

α1-CB3 (peak 5). Moreover, the peptide of peak 4 of DC is different in composition from that present in peak 4 of IC.

We can find no evidence for heterogeneity of the α1-CB3 peak from soluble collagen. If, as Miller suggests, α1-CB3 (I) and (III) demonstrate the operation of different genes, then the SC must represent protein synthesized at the same time by a single population of fibroblasts. IC and DC, on the other hand, are probably synthesized over a wider period of time and are not as homogeneous, reflecting small changes in genetic expression in the fibroblast population. The tissue specific nature of these changes is obvious.

In addition to the chain heterogeneities, another source of tissue difference is in the associated non-collagen moieties. Both IC and DC do not go completely into solution after CNBr treatment. Some collagen molecules seem to be firmly associated with an acidic protein moiety. In the case of dentin collagen, the high content of aspartic acid and serine in the CNBr residue, as well as a high content of phosphate in the residue, show that the residue contains the collagen bound phosphoprotein previously described by Veis and Perry (9).

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