COLLAGEN CROSSLINKING: ISOLATION OF TWO CROSSLINKED PEPTIDES INVOLVING α2-CB(3–5) FROM BOVINE SCLERAL COLLAGEN

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1. Introduction

Collagen, the major structural protein in the eye, as elsewhere, is responsible for giving the eye its unique and functional shape. Collagenous tissues are strengthened by the covalent crosslinks between the collagen molecules. The approximate positions of several crosslinks are known [1]. In degenerative myopia, the sclera appears thinned with loss of organisation in the scleral fibres. This change in fibrillar structure may be due to changes in collagen crosslinking and we have found two crosslinked peptides in mature bovine scleral collagen after reduction with tritiated potassium borohydride and cleavage with cyanogen bromide. These crosslinked peptides both involve the peptide α2-CB(3–5), the largest cyanogen bromide fragment, and are intermolecular. The first is a dimer of fragment α2-CB (3–5) while the second consists of fragment α2-CB (3–5) joined to one or more small peptides, similar to the crosslink found in bovine cornea [1].

2. Methods and results

Bovine eyes were obtained from A. G. Hedges (Abingdon, Berks.) as soon as possible after death. The sclera were dissected out and scraped vigorously to remove retinal, conjunctival and choroidal layers.

After washing with distilled water, they were blotted and stored at −20°C. The collagen crosslinks were reduced, stabilised and labelled with KB^3H_4 as in [2]. The collagen was purified and digested with CNBr by the method in [1]. Polyacrylamide gel electrophoresis was performed by the method in [3] as described [4].

Measurement of radioactivity and amino acid analysis were carried out as in [1].

CM-cellulose chromatography was by the method of Panjwani and Harding [4] except that a column temperature of 43° was used.

Amino acid analysis of peptides separated by SDS–gel electrophoresis was essentially by the method in [5], without prior incubation with mercaptoethanol. Determination of the radioactivity incorporated into peptides separated by SDS–gel electrophoresis was achieved by excision of the stained bands into 200 μl of 30 vol. H_2O_2, at 60°C for 2 h, or overnight at 20°C if necessary. Scintillation cocktail was then added, and radioactivity determined as in [1].

2.1 *Isolation of an α2-CB (3–5) dimeric crosslinked peptide*

CM-cellulose chromatography of the cyanogen bromide digest of labelled scleral collagen gave an elution pattern typical of type I collagen, similar to that from the cornea [4]. The last peak eluted from the column contained the majority of the radioactivity, and by SDS–gel electrophoresis was shown to be a complex mixture containing the peptide α2-CB (3–5). ^3H-labelled bands of molecular weight larger than α-chains were obtained from this fraction by SDS–gel electrophoresis, and had similar mobilities to those seen when the whole digest was examined This fraction was then subjected to chromatography at 20°C on Bio-Gel P300, 100–200 mesh (Bio-Rad Laboratories, Caxton Way, Watford, Herts) and eluted with 1 M $CaCl_2$ in 50 mM Tris–HCl (pH 7.5) as

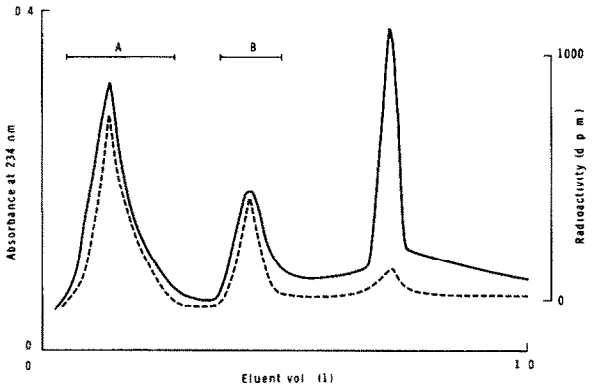

Fig 1a

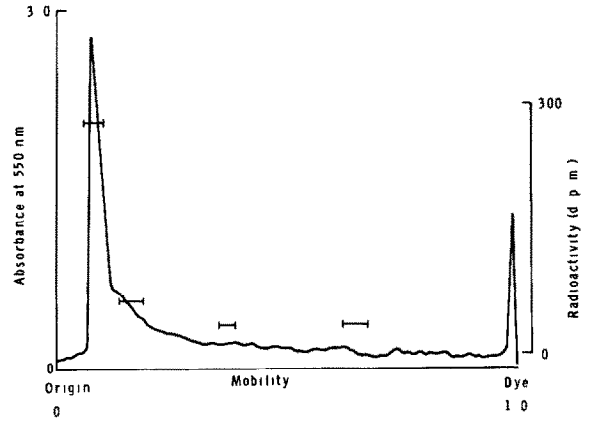

Fig 1b

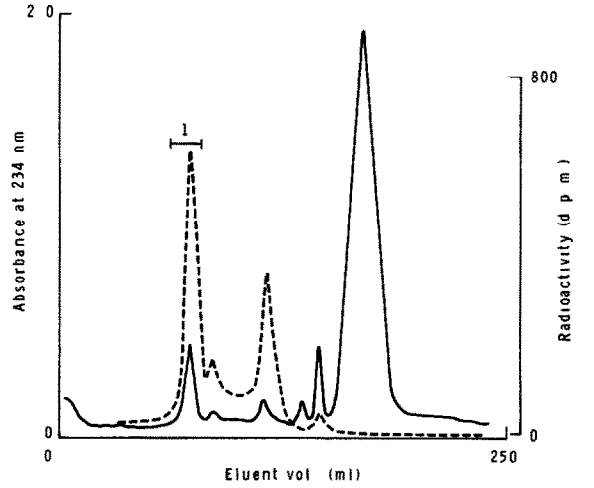

Fig 1c

shown in fig.1a. Fractions corresponding to peak A, which was radioactively labelled, containing 60% of the total counts, were pooled, freeze dried and subjected to SDS–gel electrophoresis and amino acid analysis. Figure 1b shows the staining profile of the gel after SDS–gel electrophoresis, and the single radioactively labelled band, with a mobility corresponding to mol. wt 123 000 (determined by SDS–gel electrophoresis with other CNBr peptides and α chains as markers) gave the amino acid composition of α2-CB (3–5) isolated from bovine skin as shown in table 1, both from hydrolysis of the peptide fraction, and from elution of the SDS–gel band. Note especially the absence of homoserine, which was absent even on gross overloading of the amino acid analyser. This molecular weight is almost double that of α2-CB (3–5) (60 751) determined [6], indicating that this pure peptide is a radioactively labelled crosslinked dimer of α2-CB (3–5) Short periodate digestion by the method in [7] followed by chromatography on Sephadex G-10 [1] yielded a radioactively labelled peak which after freeze-drying and SDS–gel electrophoresis gave a band which had the mobility of α2-CB (3–5).

2 2. *Isolation of a crosslinked peptide involving α2-CB (3–5) and small CNBr peptides*

After chromatography on Bio-Gel P-300 as above,

Fig 1 Separation of crosslinked peptides from bovine scleral collagen

(a) Gel chromatography of the last fraction from the CM-cellulose column on a column (130 × 3 cm) of Bio-Gel P-300 (100–200 mesh) eluted with 1 M $CaCl_2$ in 50 mM Tris–HCl (pH 7 5) at 20°C (———) A_{234}, (- - - -) radioactivity in dpm from 0 1 ml aliquots of column fractions The fractions pooled for peaks A and B are shown by the bar lines

(b) Coomassie blue staining profile of an SDS–gel obtained after electrophoresis of peak A, after dialysis and freeze drying The short horizontal bars indicate the radioactivity (dpm) in portions of the gel treated as described in the text

(c) Gel chromatography of peak B digested with HIO_4 as described in the text for 10 min A column (68 × 2 cm) of Sephadex G-10 eluted with 100 mM acetic acid was used (———) A_{234}, (- - - -) radioactivity in dpm from 0 1 ml aliquots of column fractions The fractions pooled to give peak 1 are shown by the bar line

Table 1
Amino acid compositions of crosslinked peptides from bovine scleral collagen, involving fragment α2-CB (3–5) residues/peptide

Amino acid	α2-CB (3–5) from bovine skin[a]	Eluted gel band from fig.1b	Peak A from fig.1a[b]	Peak B (fig.1a) before HIO_4 digestion	Peptide after 5 min HIO_4 digestion	Peptide (peak 1, fig 1c) after 10 min HIO_4 digestion
Hydroxyproline	51.4	51.3	54 7	55 0	55.0	54.0
Aspartic acid	30.9	30.9	30.9	29.3	29 1	29 3
Threonine	10.9	12.0	11 3	10 9	11.1	13 0
Serine	20 9	21.6	20.4	20.9	22 0	19.6
Homoserine	0.0	0 0	0 0	2.0	1 0	0 0
Glutamic acid	44.0	46 9	45.8	44 1	44 9	49.0
Proline	69 9	79 8	78.0	72.1	74.0	72 4
Glycine	211 8	203 9	206 0	201.0	197 0	202 5
Alanine	63.8	66.1	66 2	72.0	74 0	69 0
Valine	16 9	14 8	14.9	13.1	15.5	16 1
Methionine	0 0	0.0	0.0	0.0	0.0	0 0
Isoleucine	9 9	9 3	9.3	9.1	8.5	8 1
Leucine	18 9	16 7	17.0	18.6	17.9	19.5
Tyrosine	1 5	0 7	0 6	2.3	2.0	2.0
Phenylalanine	8 6	8.7	8.4	8.6	9 2	7.0
Hydroxylysine	6.6	4 9	4.4	5 1	4.4	4.7
Histidine	5 7	4 5	4 5	6 3	6.3	5 7
Lysine	11 9	12.4	14 2	14.2	13 5	12 3
Arginine	33 9	31 5	33 6	34 2	32 2	32.2
Total	618	616	620	619	617	616

[a] from [8]
[b] Mean of 3 determinations

the fractions corresponding to radioactively labelled peak B, (containing 30% of the total counts) were dialysed, freeze dried, and subjected to amino acid analysis. The resulting composition, shown in table 1, is almost identical to α2-CB (3–5) isolated from bovine skin except for the presence of homoserine. The peptide showed a radioactively labelled band on SDS–gel electrophoresis which had the mobility of α2-CB (3–5). After a 10 min digestion with HIO_4 as in [7], and chromatography on Sephadex G-10 [1] a complex elution pattern was obtained (fig.1c). Peak 1, with the identical elution volume of the untreated peptide had the amino acid composition of α2-CB (3–5), (table 1) showing that the 2 homoserine residues/peptide had disappeared. After only 5 min digestion, the amino acid composition of a similar peak after G-10 chromatography indicated the presence of 1 homoserine residue/peptide (table 1). No alterations in the mobility of the labelled band could be detected on SDS–gels. All attempts to identify the other radioactively labelled peaks eluted from the Sephadex G-10 column proved unsucessful. Presumably these other peaks must represent the small peptides joined originally to α2-CB (3–5). The amount of homoserine (2 residues/peptide) in the uncleaved peptide indicates the presence of 2 small peptides in the labelled crosslinked peptide. After 5 min digestion, 1 homoserine residue has been lost, indicating the cleavage of one of the small peptides. After 10 min digestion, no homoserine is seen in the amino acid composition, even after overloading, so both peptides have been cleaved from the α2-CB (3–5) fragment.

3. Conclusions

We have isolated 2 types of crosslinked peptide from bovine scleral collagen. The first type was shown by SDS–gel electrophoresis, amino acid analysis and HIO_4 digestion to be a crosslinked dimer of α2-CB (3–5). This must be an intermolecular cross-

link because there is only one α2-CB (3–5) fragment per molecule. The second type was shown by similar techniques to involve α2-CB (3–5) joined to possibly 2 small peptides from the homoserine content of the uncleaved peptide and is similar to the crosslink found in the bovine cornea [1]. These are the first cross-linked peptides to be isolated from scleral collagen When the amino acid sequence of fragment α2-CB (3–5) is published we will be able to pinpoint the exact sites of crosslinking and relate them to the staggered arrangement of collagen molecules.

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