ISOLATION AND PARTIAL IDENTIFICATION OF A CROSSLINKED PEPTIDE FROM BOVINE CORNEAL COLLAGEN

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1. Introduction

Collagenous tissues are strengthened by the covalent crosslinks between the molecules. The approximate positions of several crosslinks have been determined, usually by examination of CNBr digests of collagen reduced by borohydride. The first intermolecular crosslink peptide isolated in this way was from borohydride-reduced cartilage [1]. The crosslink peptides isolated from type I collagen all involve the carboxy-terminal peptide α1CB6 [2–7]. In the present paper the isolation of a crosslink peptide from CNBr digests of bovine corneal collagen is described. The peptide consists of α2CB(3–5), the largest CNBr fragment, joined to one or more small peptides.

2. Methods and results

For measurement of radioactivity portions of column effluent (0.1 ml) and of pooled fractions were made up to 0.8 ml with water and added to 9 ml scintillation cocktail (50 mg 1,4-di(2-(5-phenyloxazolyl))-benzene and 2.5 g 2,5-diphenyloxazole in 500 ml toluene and 250 ml Triton X-100). The samples were then counted in a Beckman LS-100C scintillation counter.

Polyacrylamide gel electrophoresis was by the method in [8] as in [9].

For amino acid analysis proteins were hydrolysed by 6 M HCl for 18 h at 108°C in vacuo. Hydrolysates were analysed on a Locarte amino acid analyser.

2.1. *Isolation of a large crosslinked CNBr peptide*

Adult bovine corneae were obtained from the slaughterhouse, (A. G. Hedges, Abingdon) and stored at −20°C. Both sides were scraped thoroughly with a scalpel to remove the epithelium, endothelium and Descemet's membrane before the corneae (5 g wet wt) were suspended in 500 ml 0.1 M sodium phosphate, pH7.4. The collagen crosslinks were reduced, stabilized and labelled using NaB^3H_4 [10]. The reduced corneae were washed on a nylon mesh with distilled water, cup up finely with scissors and extracted with 75 ml 4 M guanidinium chloride–50 mM Tris–HCl, pH 7.5, with shaking for 24 h at 20°C (5 changes) to remove non-collagenous material [11]. The collagen was washed with water until free of salts (tested with $AgNO_3$), and digested with CNBr [9]. Almost all (90%) of the digest was soluble in the starting buffer for the carboxymethyl cellulose chromatography.

Portions (100–500 mg) of the soluble CNBr peptides were fractionated on a column (10 × 2.3 cm) of CM-cellulose (CM-52 micro-granular from Whatman, Maidstone) using gradient elution at pH 3.6 [12]. The chromatograms obtained were similar to those found for unreduced bovine corneal collagen [9]. The portion of the column effluent corresponding to fraction 10 (see [9]) was pooled; duplicate 0.1 ml samples were taken for measurement of radioactivity before the rest was dialysed and freeze-dried. This last material leaving the ion-exchange column was about 30% original column load but had about 60% activity.

The highly labelled material was subjected to gel chromatography on a column (111 × 1.2 cm) of

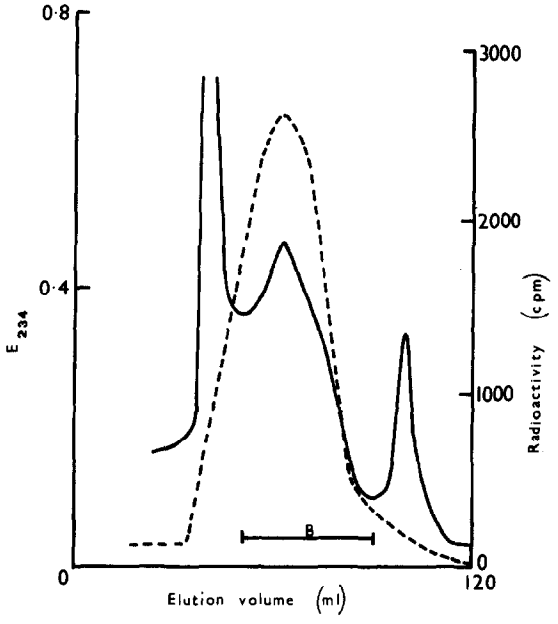

Fig.1. Gel chromatography of the last fraction from the CM-cellulose column on a column (111 × 1.2 cm) of Sepharose 6B eluted with 1 M $CaCl_2$ in 50 mM Tris–HCl, pH 7.5 at 16 ml/h (————) E_{234}; (– – –) cpm in 0.1 ml portions of column fractions. The bar shows the portion of column effluent pooled in this experiment to give fraction B.

Table 1
Amino acid composition of fractions B and B1[a]

	B[b]	B1[c]	α2CB(3–5)[d]
Hyp	85	95	92
Asp	50	48	47
Hse	2.7	0	0
Thr	17	19	17
Ser	34	36	33
Glu	69	76	70
Pro	117	129	119
Gly	340	323	345
Ala	114	108	107
Val	25	24	24
Met	0	0	0
Ileu	14	14	14
Leu	27	29	29
Tyr	4.4	3.0	2.7
Phe	13	14	14
Hyl	10	6.8	11
Lys	19	18	18
His	7	6.3	5
Arg	51	51	51

[a] Results are expressed as residues/1000 residues
[b] Mean composition of 4 preparations
[c] Mean composition of 5 preparations
[d] Isolated from CNBr digest of unreduced corneal collagen (J.J.H. unpublished result). This composition is almost identical to that reported for α2CB(3–5) of bovine corium collagen [14]

Sepharose 6B (Pharmacia, London) eluted with 1 M $CaCl_2$ in 50 mM Tris–HCl, pH 7.5, at 16 ml/h (fig.1).

Most of the activity and dry weight were recovered in fraction B with an elution volume of about 66 ml, which is the elution volume we have found for α2-CB(3–5) isolated from unreduced digests of bovine cornea on the same column. Fraction B gave a single band after sodium dodecylsulphatepolyacrylamide gel electrophoresis. The amino acid composition of fraction B is similar to that of α2CB(3–5), the largest fragment usually found in CNBr digests of type I collagen, but it contains some homoserine (table 1). The composition shows that it is largely α2CB(3–5); the high activity indicates that it is a crosslink peptide, and then the elution position from agarose and mobility on gel electrophoresis show that a single α2CB(3–5) must be joined to small peptide(s). The small peptide(s) would account for the homoserine of fraction B, and may also be responsible for the slightly high values for tyrosine and histidine. The amount of homoserine (1.8 residues/chain) indicates that two small peptides may be joined to each α2CB(3–5) fragment.

2.2. *Cleaving the crosslinks of fraction B*

To identify the small peptide(s) attached to α2CB(3–5) in fraction B, the crosslinks were split using HIO_4, and the released peptides stabilized and labelled further by reduction with NaB^3H_4. Treatment with HIO_4 in early experiments was as in [10] but later a shorter (5 min) procedure [13] was used. The periodate-treated and reduced product was fractionated by gel chromatography on Sephadex 6B, Sephadex G-10, Sephadex G-15 and Sephadex G-50 in different experiments to isolate the two parts of the crosslink peptide. In all experiments two components (B1 and B2) were isolated. The elution position of B1 from Sepharose 6B, the mobility of the single band it gave on sodium dodecylsulphate–polyacrylamide gel electrophoresis, and its amino acid

composition (table 1), show that it is α2-CB(3–5). The absence of homoserine shows that the crosslink was cleaved, and its unaltered position on Sepharose 6B confirms that the peptide(s) joined to α2-CB(3–5) are small. The low value for hydroxylysine is presumably due to its destruction by HIO_4. The amino acid composition of six preparations of B2 showed it to be a mixture of peptides and all attempts to separate and identify them unequivocally have failed.

In two further experiments the crosslink was split by heating at 70°C for 1 h instead of using periodate. Chromatography of the split products on Sephadex G-10 yielded fragment α2-CB(3–5) free of small peptides, as shown by its amino acid composition, but again the small released peptides could not be identified.

3. Conclusions

In conclusion fraction B consists of fragment α2-CB(3–5), a 655 residue peptide lacking homoserine, joined to one or more small peptides. Fragment α2-CB(3–5) is from the carboxy-terminal end of the α2 chain of collagen and does not overlap the small peptides which are all near the amino-terminus so the crosslinks of fraction B are intermolecular, end-to-end crosslinks. Only free α2-CB(3–5) has been isolated from unreduced digests of corneal collagen [9] so the crosslinks of fraction B are labile, reducible crosslinks stabilized by the borohydride reduction. Fraction B is the first crosslinked peptide isolated from corneal collagen, and the first from any collagen to be partly fragment α2-CB(3–5).

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References

[1] Miller, E. J. (1971) Biochem. Biophys. Res. Commun. 45, 444–451.
[2] Kang, A. H. (1972) Biochemistry 11, 1828–1835.
[3] Dixit, S. N. and Bensusan, H. B. (1973) Biochem. Biophys. Res. Commun. 52, 1–8.
[4] Eyre, D. R. and Glimcher, M. J. (1973) Biochem. Biophys. Res. Commun. 52, 663–671.
[5] Becker, U., Furthmayr, H. and Timpl, R. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 21–32.
[6] Henkel, W., Rauterberg, J. and Stirtz, T. (1976) Eur. J. Biochem. 69, 223–231.
[7] Scott, P. G., Veis, A. and Mechanic, G. (1976) Biochemistry 15, 3191–3198.
[8] Furthmayr, H. and Timpl, R. (1971) Analyt. Biochem. 41, 510–516.
[9] Panjwani, N. A. and Harding, J. J. (1978) Biochem. J. in press.
[10] Bailey, A. J., Peach, C. M. and Fowler, L. J. (1970) Biochem. J. 117, 819–831.
[11] Miller, E. J. and Lunde, L. G. (1973) Biochemistry 12, 3153–3159.
[12] Epstein, E. H., Scott, R. D., Miller, E. J. and Piez, K. A. (1971) J. Biol. Chem. 246, 1718–1724.
[13] Robins, S. P. and Bailey, A. J. (1975) Biochem. J. 149, 381–385.
[14] Volpin, D. and Veis, A. (1973) Biochemistry 12, 1452–1464.