Chymotryptic and Tryptic Peptides of Fragment $\alpha 1$ -CB3 from Bovine Corneal Collagen

PINPOINTING THE SITES OF HEXOSE ATTACHMENT

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Tryptic peptides of citraconylated fragment $\alpha 1$ -CB3 and chymotryptic peptides of fragment $\alpha 1$ -CB3 of bovine corneal collagen were prepared, isolated and characterized. Their amino acid compositions were consistent with the amino acid sequence of fragment $\alpha 1$ -CB3 from calf skin collagen. Two glycoside sites were identified in bovine corneal fragment $\alpha 1$ -CB3, one of them being the first located in the overlap region of collagen. The results are related to the uniformly narrow collagen fibres found in cornea and essential for its transparency.

The extent of glycosylation of collagens appears to be inversely related to the fibre diameter (Spiro, 1969; Grant *et al.*, 1969) and it has been suggested that the extra glycosides of collagens with a relatively high glycoside content may prevent fibre thickening beyond a certain limiting diameter or prevent fibre formation completely, especially if the extra glycosides were unable to fit into the axial holes of the collagen structure (Morgan *et al.*, 1970). The fibres

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of the cornea must be fine and uniform to maintain transparency and it has been shown that rabbit corneal collagen from scar-tissue contains less glucosylgalactosylhydroxylysine than normal corneal collagen (Cintron, 1974).

In the present work tryptic peptides of citraconylated fragment $\alpha 1$ -CB3 and chymotryptic peptides of fragment $\alpha 1$ -CB3 were isolated and characterized to establish that the primary structure is the same as that for fragment $\alpha 1$ -CB3 from calf skin, and to pinpoint the glycoside sites and relate them to the three-dimensional structure of the corneal collagen.

Fig. 1. Ion-exchange chromatography on a column (100 cm $\times$ 1 cm) of Dowex AG (50 W-X4) of the tryptic digest of citraconylated fragment $\alpha 1$ -CB3 from bovine corneal collagen

Elution was at 40°C with pyridine acetate buffers as described in the text. The flow rate was 12 ml/h and 3 ml fractions were collected. The column effluent was analysed by the ninhydrin method. The bars indicate the fractions pooled.

Materials and Methods

Analytical methods

Amino acid analysis, hydroxylysine glycoside analysis and determination of *N*-terminal amino acids were as described in the preceding paper (Panjwani & Harding, 1978).

Tryptic peptides of fragment $\alpha 1$ -CB3

The preparation and characterization of fragment $\alpha 1$ -CB3 from bovine corneal collagen were described in the preceding paper (Panjwani & Harding, 1978). The ϵ -amino groups of the lysine residues of fragment $\alpha 1$ -CB3 (55mg) were citraconylated essentially as described by Dixon & Perham (1968), except that the

Fig. 2. Gel chromatography of peptide T1 on a column (75 cm $\times$ 1 cm) of Sephadex G-25

The column was eluted with 200 mM-acetic acid at a flow rate of 8.5 ml/h; 2 ml fractions were collected. The bars indicate the fractions pooled.

reaction medium was 0.5 M-potassium phosphate, pH 8.2 (Slaby & Holmgren, 1975). When alkali uptake ceased after addition of the fourth portion of citraconic anhydride (BDH Chemicals, Poole, Dorset, U.K.), the mixture was stirred for another hour at room temperature (20°C) and desalted on a column (65 cm $\times$ 1.9 cm) of Sephadex G-25 with 0.5% NH_4HCO_3 , pH 7.8, and freeze-dried. The citraconylated fragment $\alpha 1$ -CB3 was dissolved in 10 ml of 200 mM- NH_4HCO_3 /1 mM- CaCl_2 , pH 8.1 (Kang *et al.*, 1975). Trypsin [diphenylcarbamoyl chloride-treated, 1 $\times$ crystallized; Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] solution (150 μ l of 0.5% trypsin in 0.11 M-HCl) was added and the mixture incubated at 37°C. After 3 h another 70 μ l of the trypsin solution was added. After a further 3 h the digest was cooled, acidified to pH 3.25 with acetic acid, and freeze-dried.

Removal of citraconyl groups from the tryptic digest was achieved by stirring in 5 ml of pyridine acetate (0.2 M in pyridine), pH 3.1, at 25°C for 3 h.

The tryptic digest of citraconylated fragment $\alpha 1$ -CB3 was dissolved in 3 ml of water, adjusted to pH 2.5 with 6 M-HCl (Aristar, BDH Chemicals) and applied to a column (100 cm $\times$ 1 cm) of Dowex AG 50 W-X4 (-400 mesh, Bio-Rad Laboratories, Richmond, CA, U.S.A.). Elution was at 40°C with 70 ml of pH 3.1 pyridine acetate (0.2 M in pyridine), followed by a linear gradient formed from 500 ml of this buffer and 500 ml of pH 4.19 pyridine acetate (1.1 M in pyridine), followed by a second linear gradient formed from 250 ml of the pH 4.19 buffer and 250 ml of pH 5.0 pyridine acetate (2.0 M in pyridine). A portion (0.6 ml) of each fraction was evaporated to dryness, redissolved in 0.5 ml of water and 0.5 ml of ninhydrin solution (as used for amino acid analysis) was added. The reaction mixture was heated to 100°C for 20 min, cooled and the A_{570} measured. Three major peaks, T1, T3 and T5, and 12 minor peaks were obtained (Fig. 1). Fraction T1 was further fractionated on a column (75 cm $\times$ 1 cm) of Sephadex G-25 with 200 mM-acetic acid, pH 2.8, to give three components (Fig. 2).

Chymotryptic peptides of fragment $\alpha 1$ -CB3

Limited cleavage of fragment $\alpha 1$ -CB3 (30 mg) by chymotrypsin (bovine α -chymotrypsin, 3 $\times$ crystallized, Sigma Chemical Co.), was carried out essentially as described by Stark *et al.* (1971) in 6 ml of 200 mM- NH_4HCO_3 , pH 8.1. The reaction was started by the addition of 750 μ g of chymotrypsin in 750 μ l of 1.8 mM- CaCl_2 . Incubation was at 37°C for 70 min before the digest was cooled, acidified with 4 drops of acetic acid and freeze-dried. A second chymotryptic digestion was performed on 25 mg of fragment $\alpha 1$ -CB3 by the same method, except that the enzyme/

Fig. 3. Gel chromatography of a chymotryptic digest of fragment $\alpha 1$ -CB3 from bovine corneal collagen on a column (137 cm $\times$ 2 cm) of Sephadex G-50 (superfine grade)

The column was eluted at 35°C with 30 mM-sodium acetate, pH 4.4. The flow rate was 18 ml/h and 3 ml fractions were collected. The bars indicate the fractions pooled. A second digest prepared under slightly different conditions gave a similar profile.

substrate ratio was 1:50 and the reaction period was 50 min.

The chymotryptic digests were fractionated on a column (137 cm $\times$ 2 cm) of Sephadex G-50 (superfine grade) at 35°C with 30 mM-sodium acetate, pH 4.8, to give four major components (Fig. 3).

Results

The amino acid compositions of some of the tryptic and chymotryptic peptides isolated during this work are given in Tables 1 and 2. The tryptic peptides of citraconylated peptide $\alpha 1$ -CB3 had not been isolated before from any collagen. The compositions of the tryptic peptides are consistent with the sequence of peptide $\alpha 1$ -CB3 from calf skin (Fig. 4) and are arranged to show up this relationship, i.e. peptides T14 and T15 both represent the first 18 amino acids from the *N*-terminus, peptide T5 represents the next 33 amino acids and so on (Table 1). The difference between peptides T14 and T15 is that the latter has 1.6 residues of lysine, whereas peptide T14 has 0.8 residue of hydroxylysine and 0.8 residue of lysine; the presence of both is because of incomplete hydroxylation of one of the two lysine residues of this peptide. Separation of pairs of peptides with and without hydroxylated lysine has been reported before (Dixit *et al.*, 1975; Rexrodt *et al.*, 1973). Of the two lysine residues of peptides T14/T15 only the one at residue 6 is likely to undergo hydroxylation because it is in the Y position in the triplet Gly-X-Y (Fig. 4).

An alkaline hydrolysate of the other hydroxylysine-containing tryptic peptide, T1B, contained 0.41 residue of glucosylgalactosylhydroxylysine, 0.17 residue of galactosylhydroxylysine and 0.10 residue of free hydroxylysine per peptide (Table 3). The total of these is close to the 0.6 residue of hydroxylysine per chain found in the acid hydrolysate of peptide T1B (Tables 1 and 3).

The chymotryptic peptide C3 had a composition (Table 2) consistent with it representing the first 62 residues of peptide $\alpha 1$ -CB3 (Fig. 4), except that it contained 0.38 residue of leucine and that phenylalanine was slightly low. To check whether this low value was due to partial chymotryptic cleavage between residues 2 and 3, the *N*-terminal amino acid of peptide C3 was determined by dansylation. Glycine was the only *N*-terminal amino acid found, indicating no cleavage between residues 2 and 3. Analysis of an alkaline hydrolysate of peptide C3 indicated that it contained 0.1 residue of glucosylgalactosylhydroxylysine, 0.2 residue of galactosylhydroxylysine and 0.1 residue of free hydroxylysine per chain (Table 3). The total value is close to 0.46 residue of total hydroxylysine per chain found in the acid hydrolysate of peptide C3.

The amino acid composition of peptide C4 (Table 2) indicates that it represents residues 63–98 of fragment $\alpha 1$ -CB3 (Fig. 4). Thus it seems that cleavage has occurred on the carboxyl side of glutamic acid-98, although that is not a typical chymotryptic split.

Table 1. *Amino acid compositions of tryptic peptides of bovine corneal citraconylated fragment α 1-CB3 and peptides TA to TG*
 Peptides T1B, T3, T5, T7, T10, T11, T14 and T15 are those isolated in this work from the tryptic digest of citraconylated fragment α 1-CB3 from bovine corneal collagen and peptides TA to TG are those that may theoretically be obtained by tryptic cleavage of citraconylated fragment α 1-CB3 from calf skin collagen. Values are expressed as residues per peptide. 0 indicates less than 0.2 residues per peptide. a, Present work; b, Fietzek *et al.* (1972).

Reference ...	a		b	a	b	a	b	a	b	a	b	a	b	a	b
	T14	T15	TA	T5	TB	T3	TC	T11	TD	T10	TE	T7	TF	T1B	TG
Hydroxyproline	1.6	1.7	2	1.7	3	6.3	6	0	0	1.1	1	1.0	1	3.8	4
Aspartic acid	0	0	0	1.2	1	1.2	1	0	0	0	0	0	0	3.7	4
Threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Serine	0	0	0	0	0	1.9	2	0	0	0	0	0	0	0.9	1
Glutamic acid	2.0	1.9	2	3.3	3	5.9	6	0.9	1	1.0	1	0.8	1	2.1	2
Proline	1.6	1.3	1	6.4	5	4.7	4	0	0	0	0	2.6	3	0.5	0
Glycine	6.0	6.3	6	10.7	11	14.9	15	1.2	1	2.2	2	4.3	4	11.4	11
Alanine	3.2	3.1	3	5.6	6	4.8	5	0	0	0	0	0.9	1	7.0	7
Valine	0	0	0	2.0	2	1.0	1	0	0	0	0	1.0	1	0	0
Isoleucine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leucine	0	0	0	0	0	1.7	2	0	0	0	0	0	0	1.2	1
Phenylalanine	0.8	0.9	1	0	0	0.8	1	0	0	0.8	1	0	0	0	0
Hydroxylysine	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0.6	0
Lysine	0.8	1.6	2	0.9	1	0.9	1	0	0	0	0	0	0	0.5	1
Arginine	1.2	1.0	1	1.1	1	0.9	1	0.9	1	1.0	1	1.2	1	0	0
Homoserine	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	1
Total	18.0	17.8	18	32.9	33	45.0	45	3.0	3	6.1	6	11.8	12	32.2	32

Table 2. *Amino acid compositions of chymotryptic peptides of fragment α 1-CB3 from bovine corneal collagen*
 Mean of two preparations under slightly different conditions. Values are expressed as residues per peptide. 0 indicates less than 0.2 residue per peptide.

Amino acid	Peptide C3	Residues 1-62 of calf skin α 1-CB3*	Peptide C4	Residues 63-98 of calf skin α 1-CB3*
Hydroxyproline	5.4	6	4.9	5
Aspartic acid	1.3	1	1.3	1
Threonine	0	0	0	0
Serine	1.1	1	1.0	1
Glutamic acid	7.0	7	4.6	5
Proline	7.8	7	3.3	3
Glycine	20.8	21	11.7	12
Alanine	9.4	10	3.9	4
Valine	1.9	2	1.0	1
Isoleucine	0	0	0	0
Leucine	0.38	0	1.8	2
Phenylalanine	1.6	2	0	0
Hydroxylysine	0.46	0	0	0
Lysine	2.3	3	1.0	1
Arginine	2.4	2	1.3	1
Homoserine	0	0	0	0
Total	61.8	62	35.8	36

* Calculated from the sequence (Fietzek *et al.*, 1972).

Discussion

The compositions of the identified tryptic and chymotryptic peptides could be related to the amino acid sequence of fragment α 1-CB3 from calf skin (Tables 1 and 2, Fig. 4), indicating that the distribution of most, if not all, the amino acids in fragment

α 1-CB3 from bovine corneal collagen is the same as that of fragment α 1-CB3 from calf skin collagen.

Glycoside determination of the hydroxylysine-containing chymotryptic and tryptic peptides showed that about 76% of the glucosylgalactosylhydroxylysine and 47% of the galactosylhydroxylysine of

Fig. 4. Amino acid sequence of fragment $\alpha 1$ -CB3 from calf skin (Fietzek *et al.*, 1972). Peptides T1B, T3, T5, T7, T10, T11, T14 and T15 are the tryptic peptides, and peptides C3 and C4 are the chymotryptic peptides isolated from corneal fragment $\alpha 1$ -CB3.

Table 3. *Glycoside composition of peptides $\alpha 1$ -CB3, C3 and T1B from bovine corneal collagen*
Values are expressed as residues per peptide.

Peptide	Glc-Gal-Hyl	Gal-Hyl	Free hydroxylysine	Total	Total hydroxylysine from the acid hydrolysate
$\alpha 1$ -CB3*	0.54	0.36	0.32	1.2	0.90
C3*	0.10	0.20	0.11	0.41	0.46
T1B	0.41	0.17	0.10	0.68	0.60

* Mean of two preparations.

Fig. 5. *Amino acid sequences around glycoside sites in collagen*

The two upper sequences are those identified in corneal collagen in the present work. The bottom sequence shows the different amino acids found close to glycoside sites in other collagens (Butler & Cunningham, 1966; Butler, 1970; Morgan *et al.*, 1970; Aguilar *et al.*, 1973; Isemura *et al.*, 1973; Fietzek & Kühn, 1976; Isemura & Ikenaka, 1977).

fragment $\alpha 1$ -CB3 is attached to residue 129; the remainder is attached to residue 6. This is the first time that glycoside has been located in the $\alpha 1$ -CB3 region of any collagen and these are the first glycoside sites to be pinpointed in corneal collagen. Residue 6 of fragment $\alpha 1$ -CB3 is in the hole region of collagen so the glycoside there could fit into the axial holes, but residue 129 is in the overlap region well away from

axial holes and yet carries the larger proportion of the glycoside of fragment $\alpha 1$ -CB3. This is the first glycoside found in the overlap region of collagen; perhaps it contributes towards limiting the diameter of corneal collagen fibres by impeding the accretion of further tropocollagen molecules.

The sequences around the glycoside sites in corneal collagen (Fig. 5) are similar to, but not identical with, two found in calf skin (see Fietzek & Kühn, 1976). The different amino acids close to the identified glycoside sites in other collagens (Fig. 3) show that, although glycosylation takes place at specific sites along the molecules, the specificity of the enzymes involved is probably not related to the immediate sequence, but may depend on longer-range features.

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