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ISOLATION AND PARTIAL CHARACTERIZATION OF A NOVEL BASEMENT MEMBRANE COLLAGEN

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SUMMARY A guanidine-HCl extraction of lens capsule basement membrane dissolves collagenous material. This material was fractionated on an Agarose A-5M column. Fractions 1, 2 and 3 were further purified and partially characterized immunochemically and by amino acid analysis. Fraction 3 has a molecular weight of 55,000 when compared with collagen type I standard. The CNBr peptide pattern and composition of fraction 3 are different from those of α 1(IV) 95K and α 2(IV) 95K chains. The results described suggest the presence of a new chain in lens capsule basement membrane.

Collagen type IV constitutes the major component of basement membranes. Based on biochemical and biosynthetic investigations, the collagenous component of basement membrane is composed of two distinct polypeptide chains α 1(IV) and α 2(IV) with M_r of about 180,000. The work on type IV collagen has been reviewed recently (1-6). The type IV collagen molecule contains a central core major triple-helix, two non-collagenous segments NC1, NC2 and the 7S domain (7). Recent reports have established that 7S is located at the amino-terminal end, whereas NC1 constitutes the carboxy-terminal end of the type IV collagen molecule (8-9). The NC2 region is postulated to be located between the 7S domain and the major triple-helix (7).

Pepsin solubilization of lens capsule produces two distinct types of fragments belonging to α 1(IV) and α 2(IV) chains (10-12). This communication describes the isolation and partial characterization of a unique new chain from lens capsule which has not been reported earlier. This new chain has been characterized by amino acid composition, SDS-PAGE, CNBr peptide mapping and by immunochemistry.

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MATERIALS AND METHODS

Preparation of Collagen

Bovine eyes were removed at the time of slaughter and frozen at -20°C. The anterior lens capsules were dissected, cleaned and lyophilized as described previously (10). 500 mg of lyophilized capsules were suspended in 100 ml of 5 M guanidine-HCl, 0.05M Tris-HCl pH 7.6, 5mM EDTA, 1mM PMSF and 2mM NEM and were exposed to 3-4 bursts of 15-20 seconds duration under the polytron (Brinkman Instruments). These broken up capsules were extracted for 20 hrs at 4°C and centrifuged. The supernatant was decanted and the residual material was extracted again for 20 hrs. The pooled extract was dialysed versus 0.02 M ammonium bicarbonate and then lyophilized.

Column Chromatography

Molecular sieve chromatography of the lyophilized material was performed on an Agarose A-5M (BioRad Laboratories, 200-400 mesh) column (2x120 cm) using 2.5M guanidine-HCl, pH 7.6. The carboxymethyl cellulose chromatography was performed on a 1x10 cm column prepared in 0.02 M acetate - 2 M urea pH 4.8 at 44°C as described earlier (12). Fraction 1 (Figure 1) was purified on a diethylaminoethyl cellulose column (1x10 cm) using the experimental procedure described (13).

SDS-PAGE Analysis

The SDS-PAGE was performed on 10% slab gels in sodium dodecyl sulfate according to procedure (14). The CNBr digests were run on 15% gels. The gels were stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 7% acetic acid and destained in 10% methanol and 7% acetic acid.

CNBr Cleavage

The collagen samples were incubated with 25% (v/v) 2-mercapto-ethanol in 0.2 M ammonium bicarbonate (pH 7.2) for 20 hrs at 45°C for the conversion of methionine sulfoxide to methionine (15). The samples were lyophilized, redissolved in 70% formic acid and treated with twice the amount of cyanogen bromide. A cyanogen bromide peptide mixture containing 2 mg/ml was prepared and 25 µl were used for electrophoretic analysis.

Immunological Analysis

Antibodies to purified fraction 3 were raised in a rabbit by injection of 1 ml of an emulsion consisting of the antigen and complete Freund's adjuvant (1mg/ml). A booster injection containing 1 mg of the antigen in incomplete Freund's adjuvant was given after 3 weeks. Antiserum was collected after 14 days and analysed for antibody levels. The antibody levels were assayed by using the ELISA procedure as described (16-17). The procedural details have been reported earlier (18).

RESULTS

The lyophilized material recovered from guanidine-HCl extract was fractionated. The elution profile from the Agarose A-5M column is presented in Figure 1. Fractions 1 to 5 were obtained consistently.

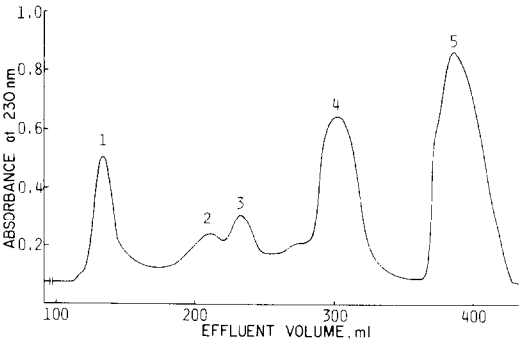


Figure 1: Molecular sieve chromatography of guanidine-HCl extracted material on Agarose A-5M column (2x120 cm). The column was equilibrated with 2.5 M guanidine-HCl, 0.02 M Tris-HCl, pH 7.6 and was eluted at a flow rate of 18 ml/hr.

This paper describes the partial characterization of fractions 1, 2 and 3 only. Fraction 1 was purified on a DEAE-52 column. The protein has a tendency to bind to the resin and was eluted with 0.1 M NaCl (figure not shown). Fractions 1 and 2 were purified on a CM-cellulose column where they eluted earlier than the elution position of α1(IV) 95K (13). The purified fractions were analysed for amino acid composition. This is presented in Table I.

The SDS-PAGE analysis of purified fraction 3 showed a homogenous single band (Figure 2). It has an apparent molecular weight of 55,000 when compared with a type I collagen standard. However, when compared with a high molecular weight globular proteins standard (BioRad Laboratories) the estimated molecular weight was found to be 95,000. The molecular weight of 95,000 is also estimated from the molecular sieve elution position of fraction 3 in Figure 1.

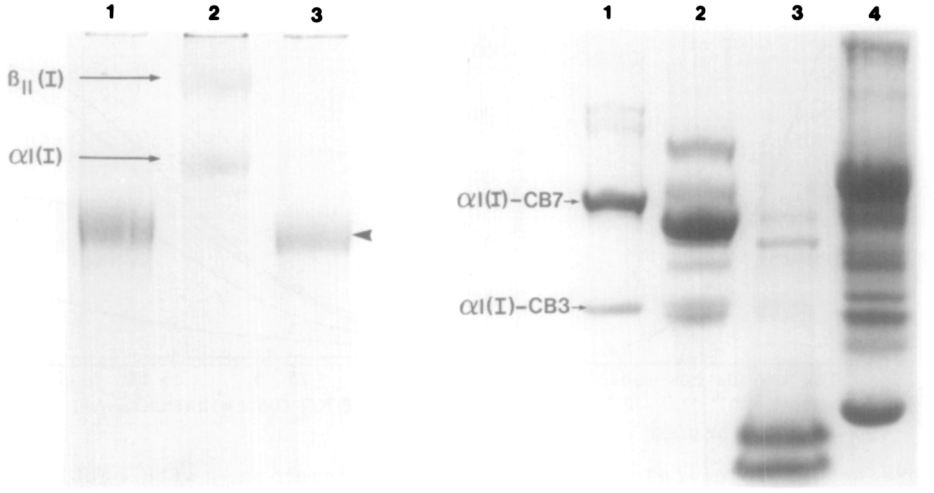
The fraction 3 and the pepsin derived chain fragments (13) α1(IV) 95K and α2(IV) 95K were cleaved with CNBr and the peptide mixtures were analysed by SDS-PAGE. As shown in figure 3, the banding pattern obtained for fraction 3 is different than the banding patterns of the α1(IV) 95K and α2(IV) 95K fragments. The low molecular weight CNBr peptides observed in fraction 3 is consistent with the high methionine content of fraction 3 (Table I).

Amino Acid Composition of Purified Fx1, 2 and 3 (Figure 1): α 1(IV) 95K and α 2(IV) 95K (calculated/1000 residues)

Amino Acid	Fx1	Fx2	Fx3	α 1(IV)95K*	α 2(IV)95K*
3-Hydroxyproline	5	6	6	8	7
4-Hydroxyproline	98	91	87	123	112
Aspartic Acid	67	54	54	50	52
Threonine	33	29	33	20	24
Serine	50	56	60	40	41
Glutamic Acid	86	78	79	82	71
Proline	82	95	96	58	55
Glycine	269	261	256	354	347
Alanine	58	63	60	35	39
Half-Cystine	10	7	9	0	2
Valine	25	26	23	26	25
Methionine	13	15	20	13	11
Isoleucine	18	20	20	25	28
Leucine	57	63	59	48	54
Tyrosine	16	9	12	3	3
Phenylalanine	29	30	30	27	37
Histidine	12	13	13	5	6
Hydroxylysine	22	37	41	52	41
Lysine	18	13	12	7	5
Arginine	38	35	32	21	41

* Amino analysis data taken from reference 13.

Antibodies were raised against fraction 3 in rabbit. High titer antiserum was obtained as assayed by ELISA. The results of serial dilutions of antiserum are shown in Figure 4. Based on these results, a dilution of 1:4,000 was used in the inhibition studies. To determine the relationship of fraction 3 to fractions 1 and 2, the antiserum was tested for cross-reactivity with fractions 1 and 2. As shown in Figure 5, the inhibition curves obtained with fractions 1 and 2 were identical with fraction 3 (control antigen) thus showing complete cross-reactivity. Antiserum, when tested with α 1(IV) 95K and α 2(IV) 95K, showed a weak cross-reactivity indicating some structural similarity or a minor cross-contamination by these



(2) (3)

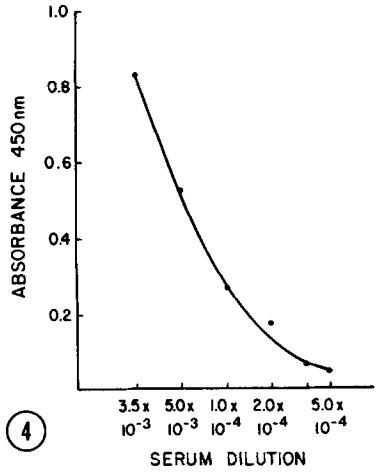
Figure 2: SDS-PAGE of fraction 3 from figure 1. Track 1, reduced fraction 3; track 2, chick skin type I collagen showing α 1(I) and ß11(I) bands; track 3, unreduced fraction 3. The arrow indicates the mobility of fraction 3.

Figure 3: SDS-PAGE analysis of CNBr digest of various collagenous components. Track 1, standard consisting of α 1(I)-CB7 and α 1(I)-CB3 of chick skin α 1(I) chain; track 2, α 1(IV) 95K; track 3, fraction 3; track 4, α 2(IV) 95K.

fragments in the purified fraction 3. No inhibition was observed using bovine 7S (8) or non-collagenous carboxy-terminal globular region NC1 (7).

DISCUSSION

This communication describes the collagenous material extracted (4-5%) by 4.5 M guanidine-HCl from lens capsule basement membranes. The fractions 1, 2, and 3 obtained after A-5M column chromatography (Figure 1) were further purified and were characterized immunochemically and for amino acid composition. Fraction 3 was characterized further to show that it is a new chain, different from the α 1(IV) 95K and the α 2(IV) 95K and the α 1(IV) 55K chains which have been documented so well (3-6). α 1(IV) 55K is the fragment derived by pepsin cleavage of α 1 (IV) 95K (18). It is different



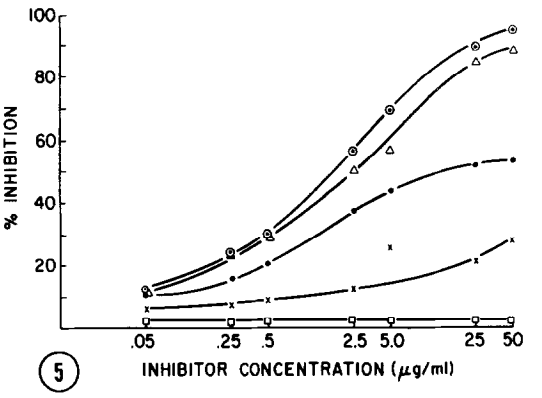


Figure 4: Immunological (ELISA) reaction of fraction 3 with rabbit antiserum raised against fraction 3.

Figure 5: Inhibition of the immunological (ELISA) reaction by fractions 2 and 3, —⊙—⊙— ; fraction 1, —△—△— ; mixture of α 1(IV) 95K and α 2(IV) 95K components, —●—●— ; NC1 domain, —x—x— 7S domain, —□—□— .

from the fraction 3 described here according to amino acid analysis and CNBr peptide mapping. The apparent molecular weight of fraction 3 from SDS-PAGE is 55,000. As determined by using globular proteins standards and its elution position on A-5M, fraction 3 would have a M_r of 95,000.

The amino acid composition of fractions 1, 2 and 3 are similar indicating a close relationship to each other. The glycine content is less than 1/3, suggesting high proportions of non-collagenous sequences in the structure. There are substantive differences in the amino acid content of proline, hydroxyproline, threonine, serine, alanine, cystine, methionine and tyrosine when compared with α 1(IV) 95K and α 2(IV) 95K (Table I). A comparison of CNBr peptide patterns of α 1(IV) 95K, α 2(IV) 95K and fraction 3, presented in figure 3, shows that fraction 3 is different from each. The low molecular weight peptides obtained from fraction 3 (Figure 3) are consistent with the high content of methionine residues.

The immunochemical results show significantly similar structural antigenic determinants in fractions 1, 2 and 3. No cross-reactivity with antiserum to either amino-terminal 7S or to the non-collagenous carboxyl-terminal domains of type IV collagen was found, thereby ruling out the possibility that fraction 3 is derived from either of these domains. Weak reactivity to a mixture of α 1(IV) 95K and α 2(IV) 95K may point to similar structural determinants or to the purity of the antigen or the inhibitor.

The results presented here show that we have isolated and characterized a new collagen chain from lens capsule basement membrane. As the amino acid composition of this chain is typical of a basement membrane collagen, the chain could be designated as α 3(IV) 55K or a new collagen, type XI, in basement membrane of lens capsule. Although present in only small amounts (4-5%), it may play a critical role in structure and organization of basement membrane collagen. This new collagen may act as an anchor between the proposed network of type IV collagen molecules, maintaining their structural integrity.

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