ISOLATION OF A MACROMOLECULAR COLLAGENOUS FRACTION AND AB_2 COLLAGEN FROM CALF SKIN

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1. Introduction

Type V collagen, isolated by [1–3] has been described in different tissues [4–6]. Type V collagen was essentially extracted from placental tissue [7–9] after limited pepsin digestion. This collagen separates from the well known interstitial collagens, type I and type III, owing to its solubility in acid solution at 0.7 M NaCl. Fractionated precipitation of the acidic pepsin digest allows the isolation at 0.7–1.2 M NaCl of a minor collagenous fraction. SDS–PAGE (sodium dodecylsulfate–polyacrylamide gel electrophoresis) shows the heterogeneity of this fraction [1,9–14] since several α-chains A,B,C,D and collagenous fragments have been separated. The results change with the conditions of extraction procedure [1,7–9, 12–14] and the origin of the tissue. α_A and α_B chains (i.e., AB collagen) are defined as type V collagen but the associated form of the chains in tropocollagen is still open to discussion [8]. The recent characterization of C_1, C and D_1, and D chains [15] points out the polymorphism of basement membrane collagen initially described as type IV collagen [16]. In addition several collagenous fragments, smaller than α-chains were also described.

We have isolated from calf skin a quantitatively minor collagenous fraction. Molecular sieve chromatography of this fraction separates two families of collagenous molecules. A HMW (high molecular weight) component 350 k (350 kilo daltons) is reduced by β-ME (β-mercaptoethanol) giving 3 chains separated on SDS–PAGE. These fragments exhibited a M_r of 55 k. The second fraction contains α_A and α_B chains in a molar ratio $\alpha_A/\alpha_B = 0.5$. This communication describes a relatively simple and repeatable isolation procedure of these components from calf skin and presents the initial results of the biochemical characterization of the HMW component.

2. Materials and methods

2.1. *Extraction of collagen*

We used the method in [17] with some modifications. The calf skin was shaved and cut into pieces of 5 mm^2. Fresh tissue (1 kg) was suspended in distilled water containing protease inhibitors (10 μM phenylmethylsulfonyl fluoride and 1 mM sodium parahydroxymercuribenzoate) then washed for 24 h with 0.2 M NaCl containing the same protease inhibitors. All procedures were carried out at 4°C. The tissue was collected after filtration, rinsed with distilled water and suspended in 0.1 M acetic acid; 8 successive extractions, 24 h each with constant stirring were performed under the same conditions. The acid-soluble collagen was collected and purified [18]. The remaining tissue was suspended in 0.5 M acetic acid containing 1 g pepsin (Worthington 2650 units/mg). The limited pepsin digestion lasted 24 h at 4°C. The first pepsin extract was collected by centrifugation 10 000 × *g*, 30 min. The residual tissue was solubilized during the second pepsin digestion carried out under the same conditions.

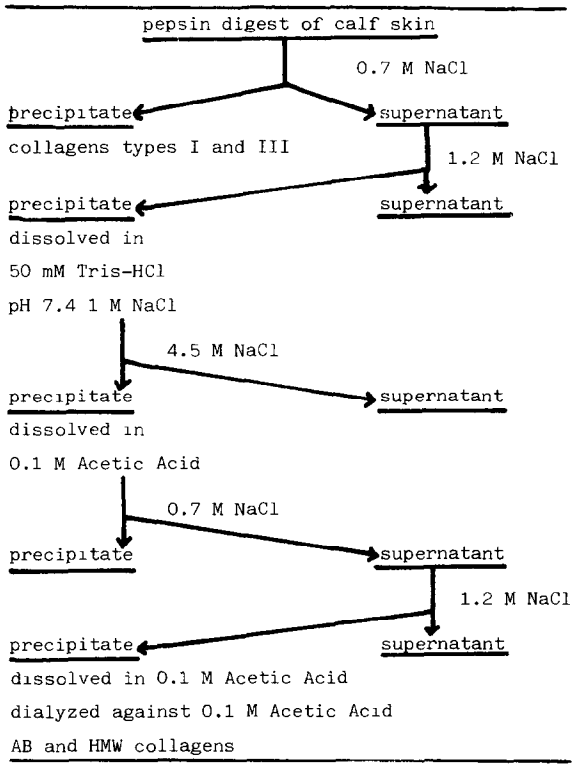
2.2. *Isolation of collagen*

The two acidic pepsin extracts were pooled and submitted to the fractionated precipitation procedure [2,19] with some modifications as presented in table 1.

2.3. *Molecular sieve chromatography*

Lyophilized samples were analyzed on different columns using in all cases the same conditions of elu-

Table 1
Procedure for the isolation of AB collagen and the HMW component



tion with 0.05 M Tris–HCl buffer (pH 7.5), 1 M $CaCl_2$. Samples (20–60 mg) were dissolved in this buffer and denatured. The effluents were collected in 5 ml fractions and the A_{230} measured (Beckman 25). The recovered components were desalted by dialysis against large volumes of distilled water, lyophilized and weighed.

2.4. *Ion-exchange chromatography*

α_A and α_B chains were separated on phosphocellulose P11 [2] and DEAE-cellulose DE 52 [8] under denaturing conditions. The column effluent was continuously monitored at 230 nm. Material was collected as above.

2.5. *Polyacrylamide gel electrophoresis*

SDS–PAGE was performed using a vertical apparatus [20,21]. Gels 160 × 120 × 2 mm contained either 5% or 7.5% or a linear gradient from 5–15% of acrylamide permitting the simultaneous analysis of the different fractions. Gels were stained and destained as in [2] and scanned at 600 nm.

2.6. *Amino acid analysis*

Samples of lyophilized components were hydrolyzed at 105°C for 24 h in 5.6 M HCl containing 0.05% β-ME. Analyses were performed in triplicate for 50, 100 and 150 μg protein as in [22].

2.7. *Determination of 3 and 4 Hyp (hydroxyproline)*

The % of collagen isolated at each step was expressed by the level of 4 Hyp determined in aliquots of each supernatant and each lyophilized fraction. Four Hyp determination was performed after 5.6 M HCl hydrolysis [23] 3 Hyp levels were determined as in [24].

2.8. *Reduction and alkylation*

The denatured HMW components obtained during Ultrogel chromatography were reduced and alkylated according to [1].

3. Results

3.1. *Recovery of collagen*

A small collagenous fraction of the acid digest remained in solution at 0.7 M NaCl and precipitated at 1.2 M NaCl. The yield from 1 kg wet calf skin was 480 mg lyophilized protein and accounted for ~0.5% of total collagen in this tissue.

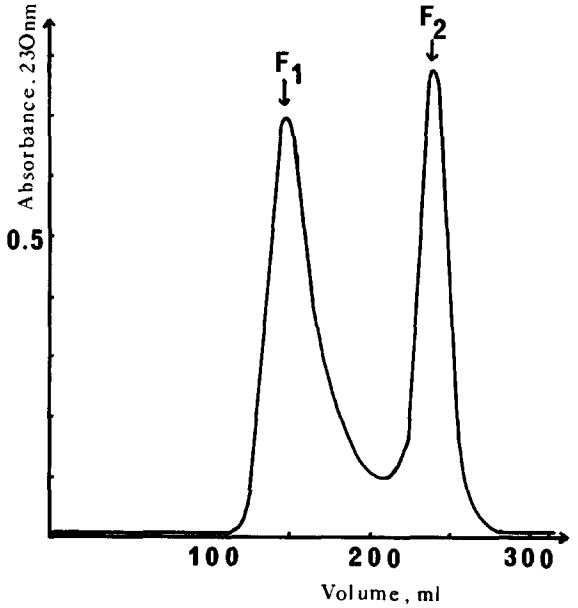


Fig.1. Chromatogram of the fraction precipitated at 1.2 M NaCl on (2.6 × 90 cm) Ultrogel ACA22 (IBF Pharmindustrie) column. Elution was carried out at a rate of 10 ml/h.

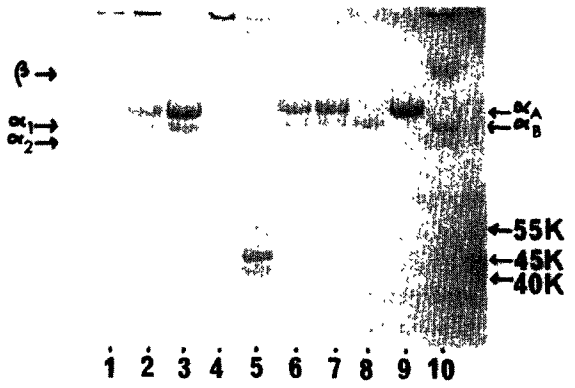


Fig.2. Sodium dodecylsulfate–polyacrylamide gel electrophoresis. The gel contains a linear gradient (5–15%) of acrylamide: (1,10) type I collagen from calf skin; (2) the whole fraction precipitated at 1.2 M NaCl; (3) the same fraction reduced by β-ME; (4) first fraction (F1) eluted on Ultrogel; (5) same fraction reduced by β-ME; (6) second fraction (F2) eluted on Ultrogel; (7) same fraction reduced by β-ME, (8) α_A chains obtained by DEAE-cellulose chromatography; (9) α_B chains obtained by DEAE-cellulose chromatography.

3.2. *Molecular sieve chromatography*

Fig.1 shows the elution pattern of the molecules precipitated at 1.2. M NaCl. The first peak (F1) which comprises 55% of the total, eluted in the void volume. Its app. M_r was ~350 k as estimated on Biogel A-15 m. The second peak (F2) eluted in the same position as that of the α_1 (I) chains, indicating an app. M_r of 95 k.

3.3. *Biochemical analysis of the HMW component*

SDS–PAGE showed only one band (fig.2(4)) in a position similar to that of γ subunits of type I collagen. Reduction by β-ME resulted principally in 3 bands exhibiting an app. M_r of 40 k, 45 k and 55 k, on SDS–PAGE (fig.2(5)). The 3 chains accounted for 80% of the whole fraction as shown in fig.3(3). The proportions of each chain were, respectively, 33%, 47% and 20%. The percentages were obtained by integration of the scanning results from 5 electrophoreses of the whole reduced fraction (precipited at 1.2 M NaCl) and of the reduced fraction F1 (the SD of the determinations is ≤2%). On reduction and alkylation the HMW component gave 6 peaks on Sephacryl (fig.4). Peaks S1 and S2 contained only 20% of total 4 Hyp and showed several bands on SDS–PAGE, which were positioned between α- and γ-collagen subunits. Peak S3 comprising 75–80% of the total 4 Hyp was shown to contain the 3 bands

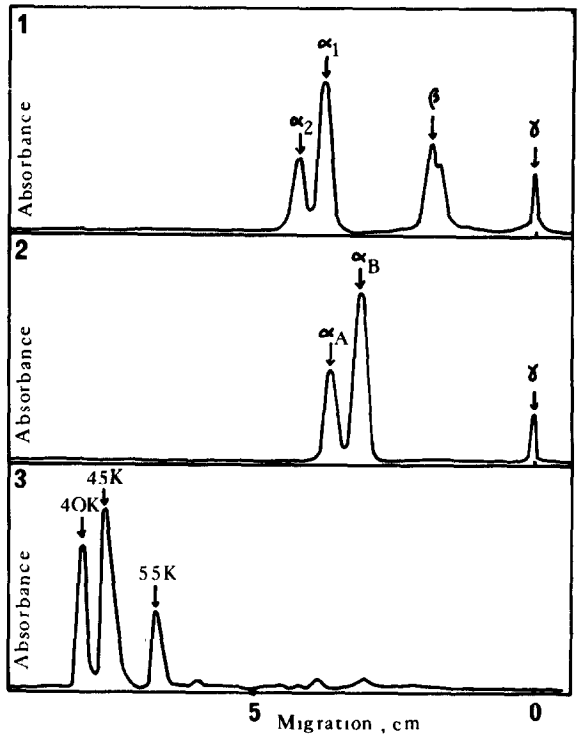


Fig.3. Spectrophotometric scans of SDS–PAGE. Gels were scanned at 600 nm, 24 h after the electrophoresis. The recorder tracings from the scans are used to calibrate the areas under the various peaks: (1) type I collagen from calf skin; (2) the whole fraction precipitated at 1.2 M NaCl; (3) first fraction eluted on Ultrogel and reduced by β-ME.

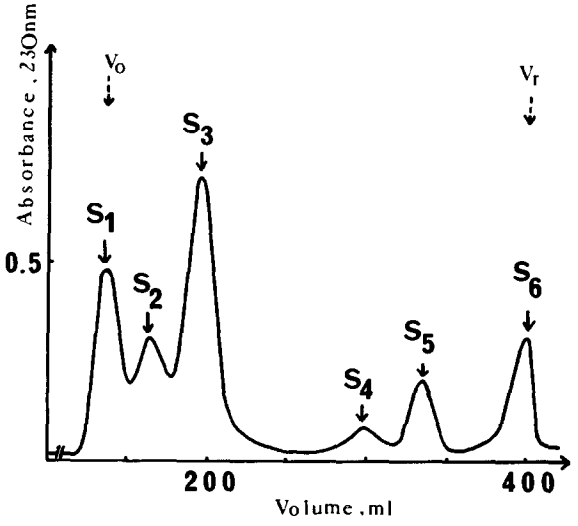


Fig.4. Separation of the reduced and alkylated HMW component on (2.6 × 70 cm) Sephacryl S 300 (Pharmacia) column. The elution was carried out at a rate of 20 ml/h. Peak S3 contains the 3 polypeptidic chains.

Table 2
Amino acid compositions of collagenous components (residues/1000)

Amino acid	Type V collagen		HMW component			
	α_A	α_B	Peak F_1	Peaks		
				S_1	S_2	S_3
3 Hyp	2	2				
4 Hyp	105	97	58	44	70	72
Asp	48	50	87	75	81	80
Thr	27	21	31	36	32	22
Ser	39	35	45	57	50	32
Glu	90	100	105	105	101	102
Pro	101	121	98	84	84	87
Gly	330	327	276	298	280	293
Ala	50	39	42	52	44	39
1/2 Cys[a]	0	0	24	12	15	22
Val	22	19	21	29	25	22
Met	12	7	11	3	15	11
Ile	14	17	28	33	30	24
Leu	38	37	27	50	39	25
Tyr	4	4	8	5	7	15
Phe	12	10	19	17	20	16
Hyl	31	50	45	35	44	59
Lys	16	18	20	18	17	19
His	10	6	3	6	2	1
Arg	49	40	52	42	44	59

[a] Calculated as *S*-carboxymethylcysteine

exhibiting an app. M_r of 55 k by molecular sieve chromatography. Peaks S4 and S5 represented small collagenous peptides containing ~5% of the 4 Hyp and were not studied. Peak S6 contained residual reagents of reduction and alkylation. Amino acid compositions of fractions F1, S1, S2 and S3 are listed in table 2.

3.4. *Biochemical characterization of the second fraction (F2)*

The fraction retarded on Ultrogel contained two chains as shown in fig.2(6). The proportion of the chains established by 12 electrophoreses is $\alpha_A/\alpha_B = 0.50 \mp 0.03$. On reduction the electrophoretic mobility of these chains on SDS–PAGE was not modified (fig.2(7)). The two chains separated on phosphocellulose (fig.5a) and on DEAE-cellulose (fig.5b). The ratio of the chains determined either by planimetry or by weighing the lyophilized material was the same $\alpha_A/\alpha_B = 0.5$. The amino acid compositions of each chain are given in table 2.

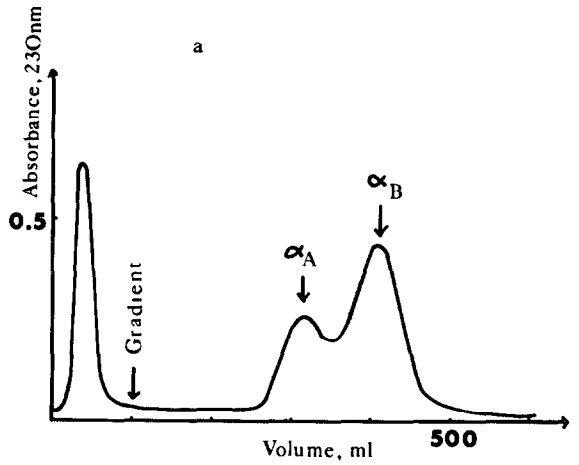


Fig.5a. Separation of α_A and α_B chains on phosphocellulose.

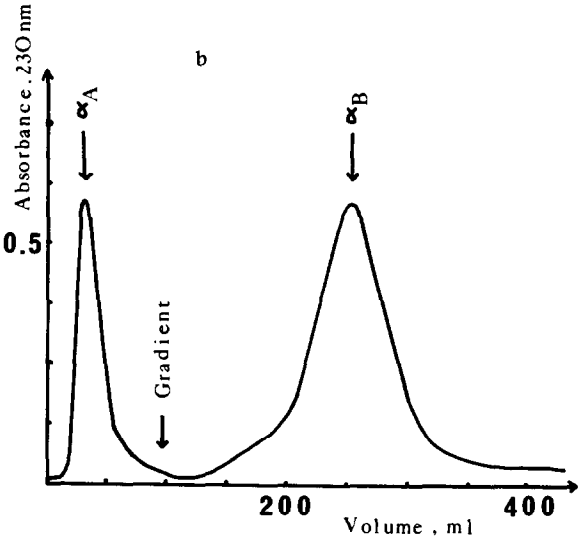


Fig.5b. Separation of α_A and α_B chains on DEAE-cellulose.

4. Discussion

The method used here to solubilize calf skin collagens moderates the action of pepsin and limits considerably the risks of proteolytic activity by the tissue enzymes. These conditions help to avoid degradation of the primary structure of collagenous molecules and this may explain the isolation of the HMW component.

The first component eluted from Ultrogel presented several particular characteristics. This type of collagenous molecule has been described in [19] from

human placental tissue. Despite the difference of tissue and species studied several similarities exist between these collagenous components. We can provide some complementary information concerning this type of molecule without discussing further the hypothesis in [19] which applies to the HMW fraction described here. The main difference with the results obtained from placental tissue is the presence of 3 chains clearly separated on SDS–PAGE instead of 2. The proportions of the 3 chains are, respectively, 2/6, 3/6, 1/6. This suggests that the 350 k molecule may be formed by an association of six 55 k subunits with disulfide linkages. The native form of the tropocollagen might consist of 3 chains. The middle of the molecule probably represents a non-helicoïdal domain and there may be an identical cleavage site for pepsin on the 3 chains resulting in an aggregate of 6 subunits. Complementary experiments are under progress to characterize completely the HMW basement membrane collagen-like component. Extraction procedures using proteases with other specificities or without enzyme at all may well be useful to clarify results concerning the structure of the basement membrane collagen.

The second type of molecules isolated were α_A and α_B chains probably associated to form $\alpha_A (\alpha_B)_2$ collagen in calf skin [8].

Acknowledgement

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