ISOLATION AND CHARACTERIZATION OF THE LARGE CYANOGEN BROMIDE PEPTIDES FROM THE α1- AND α2-CHAINS OF PIG SKIN COLLAGEN

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Received 20 May 1971

1. Introduction

Many types of soluble collagen from different sources have been reported recently, e.g., chick skin [1], chick bone [2], rat skin [3] and calf skin collagen [4]. This report is the first one dealing with the isolation and characterization of the large CNBr fragments of pig skin collagen. Pig skin collagen is very interesting from the point of view that it exhibits poor or no antigenic reactivity towards human beings [5].

2. Materials and methods

Neutral salt-soluble and acid-soluble collagens were obtained from the skin of two normal, 9 months old pigs and of a lathyritic younger animal. The latter, 3 days old animal, was made lathyritic by feeding a diet containing 0.1% β-aminopropionitrile fumarate for 3 weeks. Neutral salt-soluble collagen was extracted at 4 °C by 1 M NaCl, containing 0.05 M tris-HCl buffer at pH 7.0. Acid-soluble collagen was obtained by repeated extractions with acetic acid every time 24 hr, in 6 steps decreasing the pH values from 4.0 to 2.8. The α-components of the pig skin collagen were separated by the method previously described by Piez et al. [6].

Disc electrophoresis was carried out according to the method of Sakai and Gross [7] using a 7.5% running gel. The upper gel was prepared as described by Stark and Kühn [8].

Samples of heat denatured pig skin collagen as well as isolated α1- and α2-chains were digested with CNBr using the method employed by Fietzek and Piez [9].

The separation of the larger CNBr (CB) peptides was achieved by CM-cellulose chromatography under the conditions applied by Butler, Piez and Bornstein [3]. The purification of α1-CN4 and α1-CB5 was performed by the method from Bornstein and Piez [10]. The molecular weights of the different peptides were determined by molecular sieve chromatography on a 1.5 × 95 cm column of Biogel A 1.5 (Agarose Bio-Rad) [11].

The renaturation of the CNBr peptides was accomplished similar to the procedure used by Rauterberg and Kühn [12].

3. Results and discussion

The CM-cellulose elution pattern and the disc electrophoresis pattern of acid-soluble collagen of normal pig skin and that from skin of lathyritic pig are more or less similar to the known patterns of the other collagen species of chicken, rat and calf. The gravimetric determination of the substances obtained after desalting and lyophilization shows a ratio α1:α2 of 2:1.

In electron micrographs the cross striation pattern of the segments long spacing (SLS) obtained from the collagen of the lathyritic animal is identical with that of normal pig skin. The cross striation patterns of both fibrils and segments show a great similarity to that of calf skin collagen.

The crude opalescent pig skin collagen solution from both normal and lathyritic animals which is centrifuged by 40,000–50,000 *g* exhibits a remarkable high transition temperature of 40 °C. This is

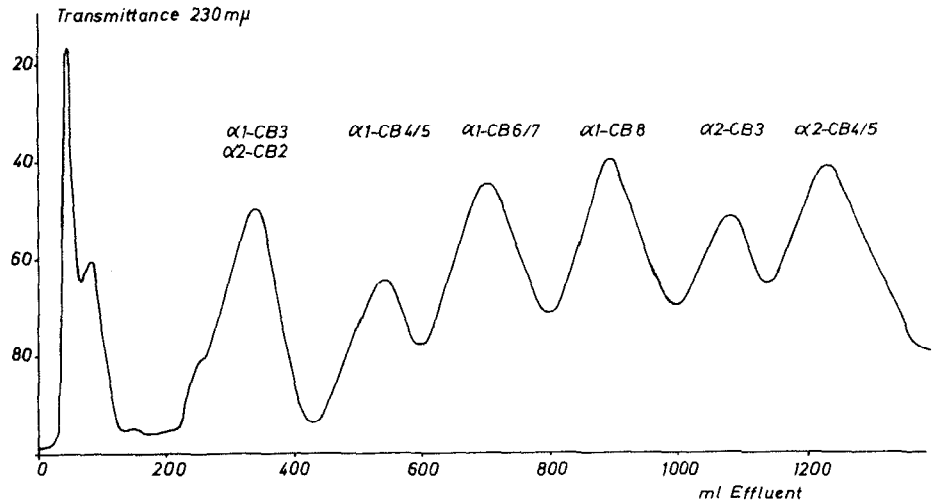

Fig. 1. Elution pattern of the CNBr peptides of pig skin collagen from CM-cellulose (Whatman microgranular CM 52) 3.2 × 15 cm with 0.02 M Na-citrate buffer at 40 °C, pH 3.7 with a linear NaCl gradient from 0.0–0.1 M and a total volume of 1600 ml.

apparently due to the presence of fatty matter, which may stabilize the structure of collagen. Centrifugation at 250,000 *g* resulted in lowering the transition temperature to 36 °C. The α1- and α2-components derived from lathyritic pig skin collagen were renatured [12] and showed transition temperatures of 31.3 °C and 23.5 °C, respectively.

Fig. 1 shows the CM-cellulose elution pattern of the large CNBr fragments derived from α1- and α2-chains.

The order corresponds to those of calf skin collagen: α1-CB3 (together with α2-CB2), α1-CB4/5, α1-CB6/7, α1-CB8, α2-CB3, α2-CB4, 5. The α1-CNBr peptides 4–7 were eluted in form of two peaks 4/5 and 6/7.

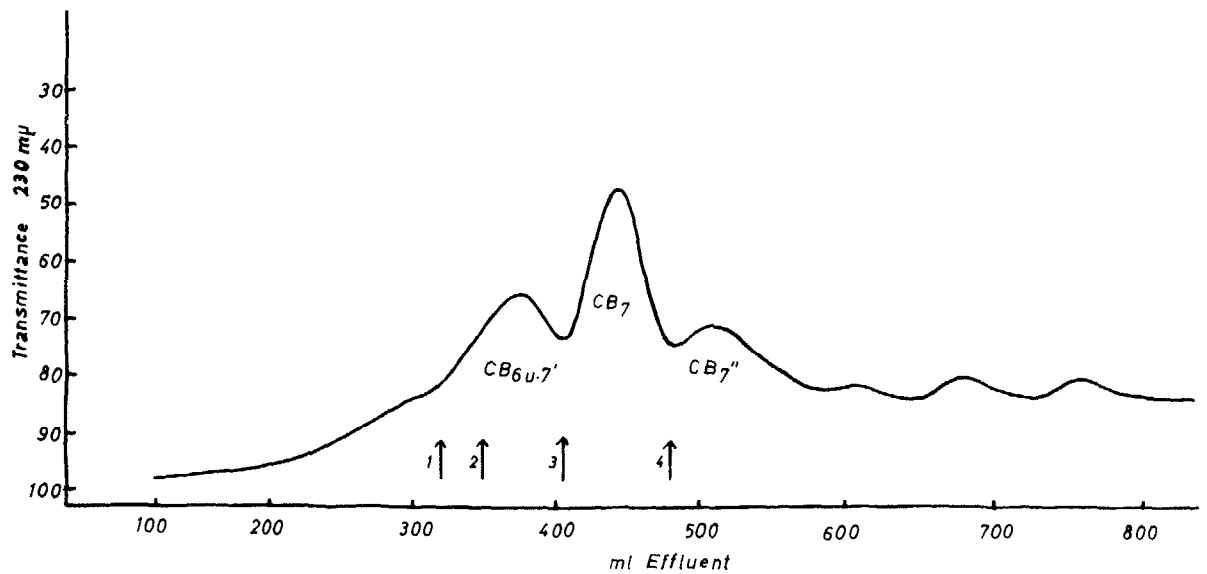

Fig. 2. Rechromatography of pig skin collagen α1-CB6 and α1-CB7 on CM-cellulose at 40 °C with 0.04 M sodium acetate buffer of pH 4.8, using a linear NaCl gradient from 0.0–0.1 M and a total volume of 1200 ml.

Table 1
Amino acid composition[a] of CB-peptides of the α1-chain of pig skin collagen.

Amino acid	α1-CB3	α1-CB4	α1-CB5	α1-CB6	α1-CB7	α1-CB8	Total CB-peptides	α1
4-Hydroxyproline	16	5 (5.4)	5 (5.4)	21	29	32	108	115
Aspartic acid	6 (5.9)	2 (2.1)	2 (2.4)	9 (8.5)	11	10	40	44
Threonine	2 (1.7)	1 (0.9)	1 (0.7)	4 (4.4)	6 (6.3)	5 (5.4)	19	18
Serine	5 (4.7)	1 (1.1)	1 (0.9)	8 (7.6)	9 (8.6)	9 (8.8)	33	37
Homoserine	1 (1.1)	1 (1.0)	1 (1.0)	0	1 (0.9)	1	5	7
Proline	15	6 (6.2)	5 (5.1)	27	34	32	119	144
Glutamic acid	16	3 (3.2)	3 (2.8)	15	17	20	74	80
Glycine	50	17	13	72	93	92	337	336
Alanine	18	6 (6.4)	4 (3.6)	25	33	34	120	115
Valine	4 (4.4)	1 (0.9)	0	4 (3.9)	4 (4.3)	5 (5.1)	18	18
Isoleucine	1 (1.4)	0	0	2 (2.3)	3 (3.0)	1 (1.2)	7	7 (7.1)
Leucine	4 (4.0)	1 (1.0)	1 (1.4)	4 (4.3)	5 (4.7)	5 (4.6)	20	21
Tyrosine	1 (0.9)	0	0	0	0	0	1	2 (1.6)
Phenylalanine	3 (2.9)	0	0	3 (2.8)	3 (3.0)	3 (3.1)	12	19
Hydroxylysine	1 (0.6)	0	0	1 (1.0)	1 (1.2)	1 (1.2)	4	5 (4.6)
Lysine	7 (6.6)	2 (1.6)	1 (1.4)	6 (6.2)	9 (9.0)	8 (8.1)	33	30
Histidine	1 (0.9)	0	0	1 (0.5)	0	0	2	2 (2.1)
Arginine	6 (6.0)	2 (2.3)	3 (2.6)	10	13	13	47	50
Total	157	48	40	212	271	271	999	1050
MW Amino acid analysis	14728	4271	3720	19241	24480	24540		
MW Agarose	14500	4500	4000	20000	25000	25000		

[a] Residues per peptide. Values are rounded off to the nearest whole number. Actual values are in parentheses in those cases where less than ten residues were found. A value of zero indicates less than 0.2 residue.

α2-CB4,5 is a unique peptide. The peptides α1-CB3, α1-CB8, α2-CB3 and α2-CB4, 5 were rechromatographed on CM-cellulose using sodium acetate buffer and NaCl gradient (see legend of fig. 2) and obtained in a homogenous state as could be confirmed by disc electrophoresis. The rechromatography of the α1-CB4/5 fraction and isolation of the peptides α1-CB4 and α1-CB5 was successful only on a phosphocellulose column with a 0.001 M sodium acetate buffer of pH 3.8 and NaCl gradient 0.0–0.3 M total volume of 1600 ml [10].

More difficult was the rechromatography of the α1-CB6/7 fraction. We succeeded indeed to get a good yield of pure α1-CB7 by pooling the fractions between the arrows 3 and 4 in fig. 2. However, α1-CB6 was always contaminated by α1-CB7. We could gain this substance only by repeated CM-cellulose chromatography with sodium acetate buffer and NaCl gradient of the substance obtained from the first part of the peak between the arrows 1 and 2.

The investigation of the small CNBr fragments is still running.

After rechromatography and desalting, the substances were rechromatographed furthermore on a calibrated Agarose column to determine their molecular weights and to purify them for the amino acid analysis. The results are reported in table 1 and table 2. The amino acid composition of the large CNBr peptides of pig skin collagen shows a great similarity to the corresponding fragments derived from rat skin and calf skin collagen.

Aliquot parts of the rechromatographed substances were renatured, dialyzed against 0.05 acetic acid and after that against 0.4% ATP solution (pH 2.8) [12]. The precipitated SLS fragments are shown in the electron micrographs of fig. 3:

a–d SLS of CNBr peptides of the α1-chain,
f–g SLS of CNBr peptides of the α2-chain
respectively correlated to an intact SLS.

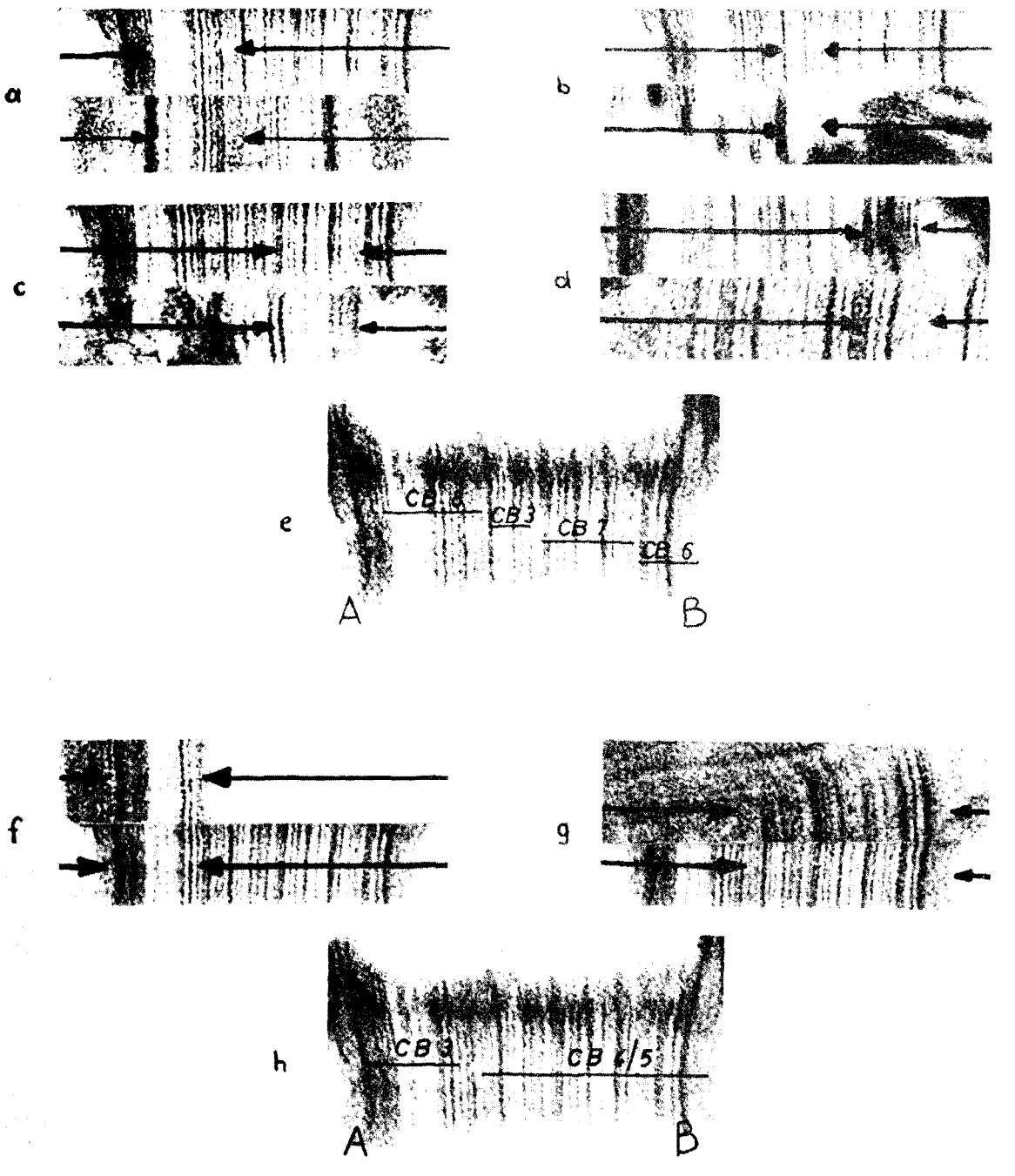

Fig. 3. Segments long spacing of CNBr peptides obtained from α1- and α2-chains of pig skin collagen after renaturation and preparation for electron microscope [12]:

(a) α1-CB8, (b) α1-CB3, (c) α1-CB7, (d) α1-CB6, (f) α2-CB3, (g) α2-CB4, 5, (e) and (h) SLS from native pig skin collagen with marks showing the order of the CNBr peptides in the α1- and α2-chains of the molecule.

Table 2
Amino acid composition[a] of CB-peptides of the α2-chain of pig skin collagen.

Amino acid	α2-CB3	α2-CB4, 5	Total CB-peptides	α2
4-Hydroxyproline	33	66	99	101
Aspartic acid	14	30	44	47
Threonine	6 (5.6)	11	17	19
Serine	9 (8.7)	25	34	34
Homoserine	1 (1.0)	0	1	4 (4.1)
Proline	37	79	116	127
Glutamic acid	22	45	67	68
Glycine	98	208	306	340
Alanine	30	70	100	112
Valine	11	18	29	36
Isoleucine	3 (3.0)	8 (8.5)	11	15
Leucine	11	15	26	32
Tyrosine	0 (0.2)	2 (1.7)	2	2 (2.2)
Phenylalanine	4 (3.6)	8 (7.9)	12	12
Hydroxylysine	3 (3.2)	5 (5.1)	8	9 (9.4)
Lysine	8 (8.0)	16	24	23
Histidine	2 (2.2)	4 (4.2)	6	8 (7.9)
Arginine	17	30	47	52
Total	309	640	949	1041
MW Amino acid analysis	28888	58906	87784	95788
MW Agarose	29000	59000		95000

[a] Residues per peptide. Values are rounded off to the nearest whole number. Actual values are in parentheses in those case where less than ten residues were found. A value of zero indicates less than 0.2 residue.

All the peptides arranged themselves end to end as dimers with symmetrical cross striation patterns.

Fig. 3e indicates, that the CNBr peptides of the α1-chain are arranged in the following order: CB8, CB3, CB7 and CB6. They begin near the *N*-terminal side of the α1-chain ending with the carboxyl terminal side. Fig. 3h shows, that the large α2-CB4, 5 fragment originates from the carboxyl end and represents 2/3 of the α2-chain. The shorter α2-CB3 fragment belongs to the amino part of the α2-chain.

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