PEPTIDE FRAGMENTS RELEASED FROM THE COLLAGEN MOLECULE BY PRONASE

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1. Introduction

A number of low molecular weight peptides could be released from the tropocollagen molecule by the action of pronase. Previous papers brought a considerable amount of evidence that these peptides should be terminal in the tropocollagen molecule (TC), as no difference was observed in electron micrographs of the Segment-long-spacing aggregates of pronase treated collagen vs. native collagen. Moreover both ultracentrifugal analysis and acrylamide gel electrophoresis revealed the presence of alpha chains only, suggesting that during the treatment with pronase the terminal cross-link region was split off [1–3]. These facts are in agreement with the results of cyanogen bromide cleavage of collagen as far as presence of cross-link in these regions is concerned [4].

However, sequencing of peptides released by pronase did not result in complete agreement with the sequences of the *N*-terminal peptides as described by Piez et al. [5]. This discrepancy prompted us to reinvestigation of our previous results [3]. There may be some differences in the sequences due to the fact that Piez et al. [5] used rat skin, rat tendon, chick skin and chick bone, whilst in our previous and present work calf skin has been used. Experimental facts presented in this paper show unequivocally that other reasons prevail in explaining the discrepancies between our telopeptide sequences published by Piez et al. [5].

2. Materials and methods

Insoluble calf skin collagen (ISC) was prepared according to Rubin et al [6]. Lyophilized ISC was subjected to solvent extraction as follows: 3 g ISC were refluxed with a mixture of 150 ml of ethanol and 50 ml of ether for 15 min; all reagents were free of water and the apparatus was protected against atmospheric moisture. The insoluble residue was filtered off and the extract evaporated to the final volume 2 ml. Insoluble residue was twice reextracted using a mixture of 200 ml chloroform and 100 ml methanol, saturated with 0.43 g gaseous HCl. Again, the whole procedure was done in absence of water. Both dinitrophenylation and dansylation revealed no increase in *N*-terminal amino acids indicating thus that no breakages occurred during this operation.

Both parts i.e. extracted ISC and peptides were subjected to pronase hydrolysis. Pronase treatment was done in 0.05 M $CaCl_2$ 20 hr at 20°. Enzyme–substrate ratio was 1:100. The resulting mixture of low molecular weight peptides was separated by dialysis, dialysate evaporated and after removal of Ca^{++} subjected to further separation as described earlier i.e. by combination of paper electrophoresis at pH = 5.6 and paper chromatography in butanol–pyridine–acetic acid–water (30:20:6:24) [3].

Peptide sequencing was done by combination of Edman degradation and *N*-terminal amino acid determination as described by Gray and Hartley [7]. Dansyl amino acids were separated by thin layer chromatography [8].

3. Results

Methanol–chloroform treatment results in releasing proteinous material from the so-called insoluble

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Table 1
The amino acid composition of the material split off by methanol–chloroform extraction as compared to the total composition of calf skin collagen and telopeptides.

Amino acid	Methanol–chloroform extracted material	Telopeptides	Total collagen
	mole/100 mole		
Glycine	28.3	25.2	32.7
Alanine	12.1	7.6	10.8
Valine	0.8	2.5	2.3
Leucine + Isoleucine	4.1	6.8	3.8
Serine	5.6	6.8	2.9
Threonine	1.6	1.6	1.9
Methionine	0.0	traces	0.5
Phenylalanine	2.3	3.8	1.5
Tyrosine	0.7	5.0	0.4
Lysine	traces	2.0	2.9
Hydroxylysine	0.0	0.5	0.5
Arginine	3.9	5.0	5.4
Histidine	4.3	1.1	0.6
Aspartic acid	17.1	6.8	4.8
Glutamic acid	11.5	8.5	7.6
Proline	5.3	11.9	13.2
Hydroxyproline	2.4	4.8	8.2

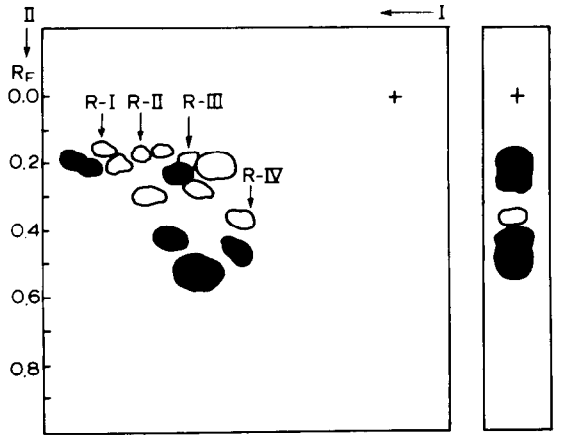

Fig. 1. Fingerprint of telopeptides split off the preextracted collagen by action of pronase (I: electrophoresis in pyridine acetate buffer, II: chromatography in *n*-butanol–pyridine–acetic acid–water). Experimental conditions and explanation of symbols see text.

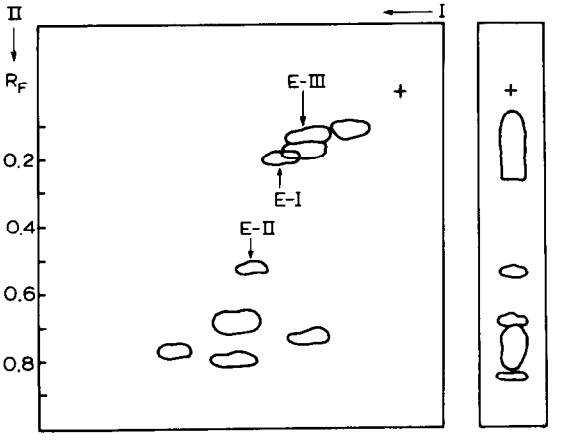

Fig. 2. Fingerprint of peptides extracted from collagen by means of ethanol–ether and chloroform–methanol–HCl (I: electrophoresis in pyridine acetate buffer, II: chromatography in *n*-butanol–pyridine–acetic acid–water). Experimental conditions and explanation of symbols see text.

collagen. The amino acid composition (table 1) resembles telopeptides only in certain aspects: likewise it is high in glycine and similarly the hydroxyproline level is reasonably low. Splitting off this material does not affect the main polypeptide chains of the tropocollagen molecule as indicated in table 1 where the results of the *N*-terminal analysis are summarized. The amount of proteinous material extractable with ethanol–ether accounts for 0.8% of total nitrogen, material extractable with chloroform–methanol accounts for 2.5% of the total nitrogen in the sample of ISC.

The peptide map of the peptides derived from the pronase treatment of preextracted ISC (see fig. 1) is much simplified compared to the crude ISC derived peptides. The amount of peptides released from the preextracted material equals 3.8% of total amino acids which is somewhat lower than in non-extracted collagen.

Out of the simplified peptide map four spots (indicated by arrows) were isolated and subjected to the sequential analysis. Henceforth the following sequences could be attributed to the four peptides isolated (the numbers show the correspondence to the *N*-terminal sequence of α_1-chain as published by Piez et al. [5]):

	4	5	6	7	
R-I	Glu.	Lys.	Ser.	Ala	
	1	2	3	4	
R-II	Gly.	Tyr.	Asp.	Glu	
	8	9	10	11	
R-III	Gly.	Val.	Ser .	Val	
	5	6	7		
R-IV	Lys.	Ser.	Ala		

Similarly the collagenous material extractable with methanol–chloroform was subjected to pronase digestion and peptide mapping. The fingerprint of this digest is presented in fig. 2. Three spots isolated from the extractable fraction were subjected to the sequential analysis. The following sequences could be attributed to the three isolated spots:

E-I	Glu.Lys.Gly.Asp.
E-II	Gly.Ala.Arg.Gly.Arg
E-III	Gly.Pro.Ala.Gly

4. Discussion

The results summarized above indicate that a considerable amount of protein material could be extracted from the insoluble collagen without splitting the main polypeptide chains of collagen. In certain aspects this material resembles the so-called telopeptides, however the amino acid composition is by no means identical with telopeptides splitt off by pronase. It becomes likely that the previously analyzed material designed as telopeptides is essentially composed of two types of peptides: those which come from the *N*-terminal region of the molecule and those which are very closely associated to the collagen structure. The evidence for this statement is presented above: from the preextracted ISC four peptides fitting into the *N*-terminal sequence of various collagens [5] were isolated whilst from the extract another three peptides fitting into the previously [3] reported sequences of telopeptides were prepared. The extractibility of this material with solvents of lower polarity seems to suggest the lipid nature of the mentioned proteinacenous material, however no definite data about its structure are currently available. It is expected that these peptides could be attributed to the presence of lipoids reported by Lampiaho et al. [9] in rat skin collagen preparations.

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