

ISOLATION AND CHARACTERIZATION OF A HIGHLY CROSS-LINKED PEPTIDE FROM ELASTIN OF PORCINE AORTA

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1. Introduction

The swine aorta contains between 40–60% of elastin (per cent of dry weight). In this protein, two particular amino acids, desmosine and isodesmosine, are present about 10 out of 1000 residues (expressed as lysine). These amino acids are responsible to a great extent for the formation of a three-dimensional network in the 'mature' elastin [1]. Other bonds, such as lysinonorleucine [2] as well as less stable precursors of desmosines [3,4], are involved in the cross-linking of elastin. All the inter and intracatenary bonds ensure the stability of the elastomer, particularly the reversion to the initial state after stretching. The number of peptide chains linked to each residue of desmosine or isodesmosine may be equal to two, three or four [5]. In order to enhance our knowledge of the peptide structure of the bridging zones, we have undertaken new investigations: first, we set up an original procedure in order to enrich the desmosine and isodesmosine contents of subfractions from enzymic and chemical digests of elastin; secondly we proceed to isolation and characterization of desmosine- or isodesmosine-containing peptides.

Abbreviations: Dansyl: 1-dimethylaminonaphthalene-5-sulfonyl; PTH: phenylthiohydantoïne; TLC: thin-layer chromatography; GC: gas chromatography.

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2. Materials and methods

Crystalline pancreatic elastase (B grade) was obtained from Calbiochem. Thermolysin and pancreas proteases (2 m units Anson/mg) were purchased from Merck. Sephadex G-25 was a product of Pharmacia. All chemical reagents were of analytical grade. Elastin was obtained after successive extractions from swine aorta according to the experimental procedure of Moschetto et al. [6]. The acidic glycoproteins which were strongly combined with elastin were eliminated by treating the elastin residue by 0.1 N NaOH during 45 min at 37°C [7].

The elastase digestion was performed in 0.05 M ammonium acetate buffer, pH 8.8, during 21 hr at 38°C with a Radiometer pH stat. The enzyme: substrate ratio was 1:100. After dilution and lyophilization the enzymic digest of elastin was submitted to gel filtration on a Sephadex G-25 column (4 × 100 cm) eluted with 0.2 N acetic acid. Only the excluded fraction was kept for further investigations. The thermolysin digestion was performed in a 0.01 M Tris buffer pH 8.0 containing 0.005 M CaCl₂ at 38°C. After the first addition of enzyme (2:100 by weight) the pH was regularly controlled and readjusted; a further quantity of thermolysin (1:100) was added after 4 hr; the total time of hydrolysis was 8 hr and the reaction was stopped by immersion in a boiling water bath. After this step, another digestion with a mixture of pancreas proteases (1:100) was performed in the same buffer at 38°C during 16 hr; it was followed by gel filtration on a Sephadex G-25 column (2.5 × 200 cm). The excluded fraction was kept for partial acid hydrolysis whereas the other fraction, containing

short peptides devoid of desmosine or isodesmosine, was discarded.

The partial acid hydrolysis was performed in concentrated HCl at 37°C during 72 hr (10–20 mg of enzymic digest mixture/ml HCl). The solution was diluted and lyophilized and then submitted to gel filtration (Sephadex G-25, column: 2.5 × 200 cm). After each chromatographic step, the effluent was monitored by UV absorption at 278 nm and the constituents were identified by the ninhydrin or Lowry reactions performed on a Technicon Auto-Analyzer. The highly cross-linked peptide was finally isolated by preparative high voltage paper electrophoresis at 2200 V in pyridine–acetic acid–water (10:100:2890) pH 3.6, followed by Whatmann 3MM paper chromatographies in solvent 1: butanol–pyridine–acetic acid–water (150:100:30:120) and in solvent 2: butanol–acetic acid–water (35:40:25). Determinations of the N-terminal residues were performed by four methods: 1) Dansylation according to Bruton and Hartley [8]; 2) Edman's degradation with direct identification of PTH–amino acids by TLC according to the experimental procedure reported by Han et al. [9]; 3) quantitative analysis of PTH–amino acids by GC according to Pisano and Bronzert [10]; 4) subtractive Edman's degradation according to Han et al. [9]. The C-terminal residues were identified by hydrazinolysis followed by amino acid analysis [11]. Amino acid analyses including desmosine, isodesmosine and lysinonorleucine were carried out with a Technicon amino acid Analyzer.

3. Results and discussion

Fig. 1 summarizes the experimental procedure of enzymic and chemical digestions of elastin and of isolation of the highly cross-linked peptide. Fig. 2 shows the elution pattern on gel filtration (Sephadex G-25) of the partial acid hydrolysate after elastase, thermolysin, pancreas proteases digestions of elastin. The subfractions F_2 and F_3 are very enriched in cross-linking amino acids, whereas the other subfractions contain free amino acids and oligopeptides, with very low contents of desmosine and isodesmosine. The fingerprints of F_2 and F_3 did show that only six peptides were present in these subfractions. We did perform column ion-exchange chromatography, in

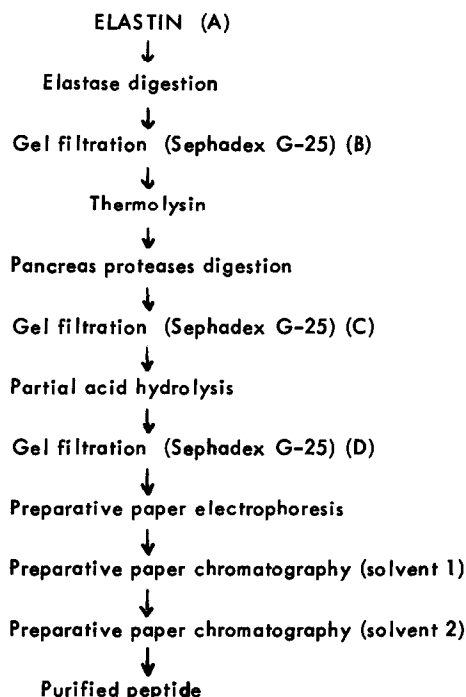


Fig. 1. Experimental procedure of enzymic and chemical digestions of elastin and of isolation of the cross-linked peptide. (A): Starting material (elastin); (B): Excluded fraction obtained after gel filtration on Sephadex G-25 of the elastase digest of A; (C): Excluded fraction obtained after gel filtration on Sephadex G-25 of the thermolysin + pancreas protease digest of B; (D): Peptide fractions obtained after gel filtration on Sephadex G-25 on the partial acid hydrolysate of C.

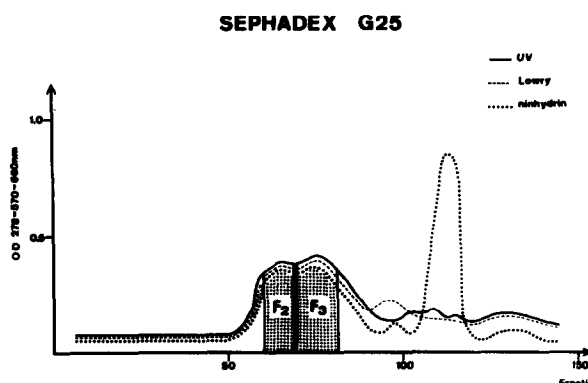


Fig. 2. Elution pattern of the partial acid hydrolysate after elastase, thermolysin and pancreas proteases digestions.

Table 1
Amino acid compositions of cross-linked peptide fraction
at different stages of enrichment, expressed in residues
per 1000

	A	B	C	D
OH-PRO	15.62	29.94	12.61	—
ASP	6.30	4.79	12.98	5.97
THR	15.93	10.92	19.37	5.05
SER	12.65	9.57	23.06	8.16
GLU	20.55	11.10	21.00	7.70
PRO	121.10	141.14	124.20	193.92
GLY	293.97	287.10	272.61	93.92
ALA	222.53	198.82	279.74	350.17
CY/2	3.18	4.58	5.99	—
VAL	128.32	180.79	54.27	33.57
MET	5.22	—	—	—
ILE	18.75	8.16	14.43	11.84
LEU	56.73	36.59	45.05	27.52
TYR	17.34	17.03	26.03	18.08
PHE	33.72	13.22	8.08	5.92
LYS	9.32	7.27	12.36	22.10
HIS	1.66	—	—	—
ARG	7.40	6.55	tr.	tr.
IDES/4	4.15	13.95	28.03	96.24
DES/4	5.92	15.47	34.48	111.05
LNL/2	0.90	2.90	5.71	8.80

order to isolate a homogeneous cross-linked peptide. In spite of the apparent simplicity of the peptide mixture, neither cation-exchange resin (Chromobeads-P, Technicon) [12] nor anion-exchange resin (Dowex 1X2) [13] did yield satisfactory results even if elution buffers of extreme pH were used. The highly cross-linked peptides were strongly retained on the Chromobeads P column and only the peptide fractions containing little desmosine and isodesmosine were eluted. Therefore, preparative paper electrophoresis and chromatographies were finally used to isolate the cross-linked peptide.

Table 1 summarizes the amino acid compositions of the whole elastin (A), of the elastase digest of elastin (excluded fraction from Sephadex G-25) (B), of the thermolysin and pancreas proteases digest of previous fraction (excluded fraction from Sephadex G-25) (C) and of the peptide subfraction F₂ obtained by gel filtration of the partial acid hydrolysate of enzymic digests (D). The cross-linking agents such as isodesmosine, desmosine and lysinonorleucine (expressed in quarter of residue for desmosine and in half

residue for lysinonorleucine) were enriched from 4; 5.9 and 0.9 out of 1000 residues of (A) to 14; 15.5 and 2.9 residues of (B) to 28; 34.5 and 6 residues of (C) to 96; 111 and 9 residues of (D). All calculations were based on 1000 residues even for (D) which are relatively short peptides.

In the first part of our work, we have set up a new method in order to enrich the desmosine and isodesmosine contents of peptide subfractions after enzymic and chemical digestions. The sum of desmosine and isodesmosine residues passed from 10 to 207 out of 1000 residues giving a yield of enrichment of 21 times at the final stage. After each step of enzymic digestion, the short peptides which provided from outside of cross-linking zones of elastin were discarded. The excluded subfraction obtained from gel filtration on Sephadex G-25 was kept. The cross-linking amino acids were present only in this fraction. Because the desmosine and isodesmosine form the cross-linking bonds in the three-dimensional network, most proteolytic enzymes do not have access to the bridging zones. There is a steric hindrance in the 'desmosine or isodesmosine core' which prevent enzymic hydrolysis of peptide bonds to form short peptides. Therefore, we have used the partial acid hydrolysis which cleaves randomly the peptide bonds including the 'desmosine or isodesmosine core'. The highest enrichment in desmosine is obtained in the F₂ subfraction of the partial acid hydrolysate (D) which is also the richest in alanine contents. This result of close association of alanine with the cross-links is in good agreement with those of Keller et al. [14], Shimada et al. [15], Thomas [16], Foster et al. [17], which were obtained on elastin from bovine ligamentum nuchae. Sandberg et al. [18,19], Foster et al. [20] showed the rich clustering of alanine residues around the lysine in small peptides of tropoelastin from aorta of copper-deficient swine which are probably involved in the formation of the desmosines cross-links of insoluble elastin.

In the second part of our work, we did isolate and characterize a highly cross-linked peptide. Its amino acid composition, expressed in molar ratio, is: Pro: 2.54 [3]; Gly: 3.05 [3]; Ala: 10.60 [11]; Desmosine: 0.94 [1]; Isodesmosine: 1.15 [1]. The number in the parenthesis is the nearest integral number of residues. The total number of residues of this peptide is 19. Because of the complex structural pattern of this

peptide, the N-terminal residues are established by four methods: 1) Dansylation (qualitative); 2) Edman's degradation with direct identification of PTH-amino acids by TLC (qualitative); 3) Edman's degradation with direct identification of PTH-amino acids by gas chromatography (quantitative); 4) Subtractive Edman's degradation with amino acid analysis (quantitative).

The C-terminal residues were established by hydrazinolysis and quantitatively analysed by Autoanalyzer.

Table 2 summarizes the N-terminal and C-terminal residues of the cross-linked peptide obtained by different approaches. The N-terminal analyses of this pep-

tide reveal the presence of 4 residues of alanine, 1 residue of glycine and the C-terminal analysis yields 3 residues of alanine and 2 residues of glycine. Therefore at least 5 side peptide chains are present in this cross-linked peptide. The most striking result is the drastic decrease of both desmosine and isodesmosine after the first step of Edman's degradation. Dansyl-desmosine and dansyl-isodesmosine are also present in aqueous phase as revealed in TLC on polyamide sheet after dansylation of the *initial* peptide [21]. After the second step of Edman's degradation, only traces of desmosine and isodesmosine are detected in

Table 2
Different experimental approaches of determination of N- and C-terminal residues of the cross-linked peptide

Amino acids composition			
of initial peptide	of residual peptide 1st step Edman degradation	of residual peptide 2nd step Edman degradation	of residual peptide 3rd step Edman degradation
Pro : 2,54 (3)	Pro : 2,64 (3) : =	Pro : 2,39 (2) : -1	Pro : 1,80 (2) : =
Gly : 3,05 (3)	Gly : 2,29 (2) : -1	Gly : 2,09 (2) : =	Gly : 2,06 (2) : =
Ala : 10,60 (11)	Ala : 6,62 (7) : -4	Ala : 4,39 (4) : -3	Ala : 4,13 (4) : =
Des : 0,94 (1)	Des : 0,30 (0) : (-1)	Des, Ides: trace	
Ides : 1,15 (1)	Ides : 0,34 (0) : (-1)		
Total n°. of residues: 19	Total n°. of residues: 12	Total n°. of residues: 8	Total n°. of residues: 8
N-terminal residues			
A) Dansylation TLC : Organic phase : Ala (4+) : Gly (+)	A) Dansylation TLC : Organic phase: Ala (3+) : Pro (+)	A) Dansylation TLC : Organic phase : Ala (3+) : Pro (+)	A) Dansylation TLC : Organic phase : Pro (2+) : Ala (+)
Aqueous phase: substituted Des and Ides			
B) PTH TLC : Ala (4+) : Gly (+)			
C) PTH GC : Ala (4) : Gly (1)	C) PTH GC : Ala (2) : Pro (1)	C) PTH GC : Ala (3) : Pro (1)	
D) Subtractive: : Ala (4) : Gly (1)	D) Subtractive: : Pro (1) : Ala (3)	D) Subtractive: : Ala (3) : Pro (1)	
C-terminal residues			
Hydrazinolysis : Ala (3) : Gly (2)			

the residual peptide. The reason is still obscure. The classical mechanism of Edman's degradation in the presence of free amino groups of several side peptide chains and of desmosine and isodesmosine might make the cleavage and cyclisation possible from the desmosine and isodesmosine rings to the adjacent peptide bonds. The real mechanism is unknown. The presence of free desmosine or (and) isodesmosine in the peptide is excluded because we did apply the initial peptide *before* total acid hydrolysis to amino acid Autoanalyzer on a column of resin Chromobeads-A and no free amino acids, neither desmosine nor isodesmosine were detected.

Another salient result is that the desmosine ring seems to be located and covalently bonded in the close vicinity of the isodesmosine ring which might be separated by only some amino acid residues. Of course, the hypothesis of the presence of 2 peptides in our peptide fraction is not entirely excluded: one might contain desmosine, the other might contain isodesmosine. However we think it is improbable because extreme precautions have been taken by us to prevent working on a mixture of peptides instead of investigating on a homogeneous peptide. All experiences are carried out and repeated 3 times. The control of homogeneity is performed by paper electrochromatography in different buffers and only one spot is revealed. The yield of isolation of this peptide is very poor because 10 steps of enrichment have been performed. From highly purified elastin (1.2 g), only 30 mg of F₂ fraction was obtained from gel filtration on Sephadex G-25 of the partial acid hydrolysate. From 30 mg of F₂, only 200 nmoles of peptide material were finally obtained after preparative electrophoresis, preparative chromatography in solvent n°. 1 and in solvent n°. 2. We did perform three steps of Edman's degradation. The amino acids established after the first step-wise degradation are only indicative. Their exact positions around desmosine and isodesmosine rings, and the amino acids which were covalently bonded between the desmosine and isodesmosine rings are also indicative. The complete and exact covalent structure of this peptide cannot be established by the present works. However, based on our experimental results and data of Gray et al. [22], we can propose a hypothetic scheme of possible covalent structure of the highly cross-linked peptide which is shown in fig. 3. It is very hard to open the pyridinium ring of desmosine or isodesmosine without cleaving the peptide

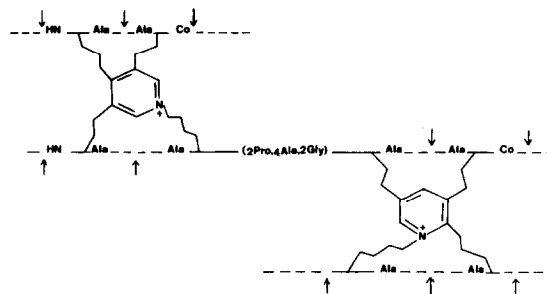


Fig. 3. Scheme of possible covalent structure of the cross-linked peptide. Arrows: Peptide bonds cleaved by enzymic and partial acid hydrolyses.

bonds. The yield is very poor and the oxidation of desmosine or isodesmosine ring is often incomplete. Large scale and very long term preparations of peptide materials are necessary. However knowledge, even partial, of the amino acids located around the bridging zones can elucidate the biochemical mechanism in which the functional properties of elastic fibers are altered during aggression, ageing or atherosclerosis.

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