Journal of Chromatography, 250 (1982) 68–72
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,168

Note

High-performance liquid chromatography of peptides obtained from elastin by alkaline hydrolysis

KHÉMARA KHAM* and BERNARD SEBILLE

Laboratoire de Physico-Chimie des Biopolymères, Université de Paris Val de Marne, Avenue du Général de Gaulle, 94010 Creteil Cedex (France)

and

ELEMER MOCZAR and MADELEINE MOCZAR

Laboratoire du Tissue Conjonctif, Institut de Recherche sur les Maladies Vasculaires, de l'Université de Paris-Val de Marne, Faculté de Médicine, 8, Rue du Général Sarrail, 94010 Creteil Cedex (France)

(Received June 28th, 1982)

Soluble peptides obtained by partial hydrolysis of cross-linked elastin were used as models for studies of structure and interactions of this protein[1,2]. One of the chemical methods for the preparation of soluble elastin degradation products is alkaline hydrolysis in aqueous alcohol[2]. The mixture of the soluble peptides obtained by this method was called kappa-elastin. It was suggested that ethanol and higher alcohols would facilitate the hydrolysis by deorganising the hydrophobic regions of elastin. It has been shown that, after hydrolysis for 18–72 h at room temperature[3], non-coacervable, desmosin-containing, low-molecular-weight (molecular weight ≈ 10,000 daltons) peptides are formed. These peptides are remarkably resistent to further hydrolysis. The peptide mixture obtained contains several populations of homologous peptides, exhibiting different characteristic glycine–alanine ratios (1.2:1, 1:2, and 1:1.2, respectively). The ratio of these peptide populations changes as hydrolysis proceeds. Earlier studies indicated that the peptide mixture obtained by alkaline hydrolysis is heterogeneous. Some peptides were partially separated from this mixture by isoelectric focusing[3]. As the most important difference between the individual peptide populations is the ratio of hydrophobic amino-acid residues, methods involving hydrophobic interactions should be more effective for the separation of the different classes of these peptides than methods based on charge differences.

To obtain more insight into the aggregation properties and the separation of the different peptide populations in kappa-elastin, we investigated the behaviour of these peptides on high-performance liquid chromatographic (HPLC) columns used for the separation of proteins.

MATERIALS AND METHODS

Preparation of kappa-elastin

Elastin from *Ligamentum nuchae*, prepared by the Lansing procedure[4], was used for the preparation of kappa-elastin[2]. Hydrolysis in 80% aqueous ethanol con-

0021-9673/82/0000–0000/$02.75 © 1982 Elsevier Scientific Publishing Company

taining 1 N potassium hydroxide was performed for 24 h at 37°C, as described elsewhere[3]. The low-molecular-weight (10,000–16,000 daltons) peptide fraction was isolated by exclusion chromatography on a Sephadex G-100 column[3].

Chromatography

The HPLC columns (7.5 cm × 0.45 cm I.D.) used for this study were filled with chemically modified LiChrospher SI 100 (Merck, Darmstadt, G.F.R.) by grafting hydrophilic diol function by a procedure previously described[5].

Solvent delivery was carried out by a Waters Model 6000 A pump, and the injector was a Waters U6K (Waters Assoc., Milford, MA, U.S.A.). Detection was performed with an Varichrom multiwave-length detector from Varian (Walnut Creek, CA, U.S.A.) at 220 nm.

The eluents were mixtures of ethanol and ammonium acetate (10^{-2} M) in different proportions. The pH of the eluent was adjusted by adding acetic acid to the solution.

Experimental conditions

Hydrolysed elastin (5 mg) was dissolved in 5 ml of the eluting solution, and 5 μl of this solution were injected onto the column.

We have studied the influence of pH, eluent composition and concentration of the sample on separation.

For the semi-preparative experiments, we used 30 × 0.45 cm I.D. columns and a sample concentration of 1 mg/ml. The eluent was ethanol–10^{-2} M ammonium acetate (60:40, v/v).

RESULTS AND DISCUSSION

The ammonium acetate solution, without the addition of alcohol, resolved the peptide mixture into two peaks at pH 7 and 8.5 (Table I). At pH 5 all the material is

TABLE I

THE pH DEPENDENCE OF THE SEPARATION OF THE LOW-MOLECULAR-WEIGHT ELASTIN PEPTIDE

Column, 7.5 × 0.45 cm I.D.; injected volume, 5 μl.

pH	*10^{-2} M ammonium acetate*		*Ethanol–10^{-2} M ammonium acetate (60:40, v/v)*	
	Elution time (min)	*Ratio of peak areas**	*Elution time (min)*	*Ratio of peak areas*
8.5	1.4	62	1.8	81
	2	38	3.3	19
7	1.4	58	1.8	82
	2	42	3.3	32
5	–	–	1.9	86
	2	100	3.3	14

* The distributions of the peak areas (absorbance at 220 nm) are expressed as the percentage of the sum.

TABLE II

DEPENDENCE OF THE ELUTION TIMES ON THE ETHANOL CONCENTRATION IN THE ELUENT

Injected volume, 5 μl; concentration, 1 mg/ml; column, 7.5 × 0.45 cm I.D.; flow-rate: 0.5 ml/min; eluent, ethanol–10^{-2} *M* ammonium acetate (v/v).

Ethanol (%)	*Retention time (min)*	*Proportion of the peak areas as a percentage of the total*
0	1.4	62
	2	38
60	1.8	80
	3.2	20
70	1.8	82
	2.9	18
80	1.8	64
	2.7	36

retained in the elution volume corresponding to peak 2 (Table I). The ethanol–aqueous solution mixture (60:40, v/v) resolves the peptides into two peaks, at all the pH values studied.

These findings may be attributed to hydrophobic interactions, similar to those participating in the coacervation phenomena. The pH optimum for the coacervation of soluble elastin peptides of higher molecular weight[1] is between pH 4.5 and 5. The low-molecular-weight kappa-elastin peptides do not form visible aggregates under the conditions of coacervation, but the formation of soluble aggregates for these sub-

TABLE III

EFFECT OF THE CONCENTRATION OF THE INJECTED SAMPLE ON THE SEPARATION OF THE KAPPA-ELASTIN PEPTIDE

Column, 7.5 × 0.45 cm I.D.; eluent, ethanol–10^{-2} *M* ammonium acetate (60:40, v/v).

Concentration (mg/ml)	*Peak number*	*Retention time (min)*	*Ratio of peak area*	*Injected volume (μl)*
1	1	1.8	62	5
1	2	2.7	38	
(1/2)	1	1.8	66	10
(1/2)	2	2.7	34	
(1/4)	1	1.8	64	20
(1/4)	2	2.7	36	
(1/8)	1	1.8	53	20
(1/8)	2	2.7	47	
(1/16)	1	1.8	55	40
(1/16)	2	2.7	45	
(1/32)	1	1.9	39	
(1/32)	1a	2.2	37	80
(1/32)	2	2.7	24	
(1/64)	1	1.9	37	
(1/64)	1a	2.2	37	80
(1/64)	2	2.7	26	

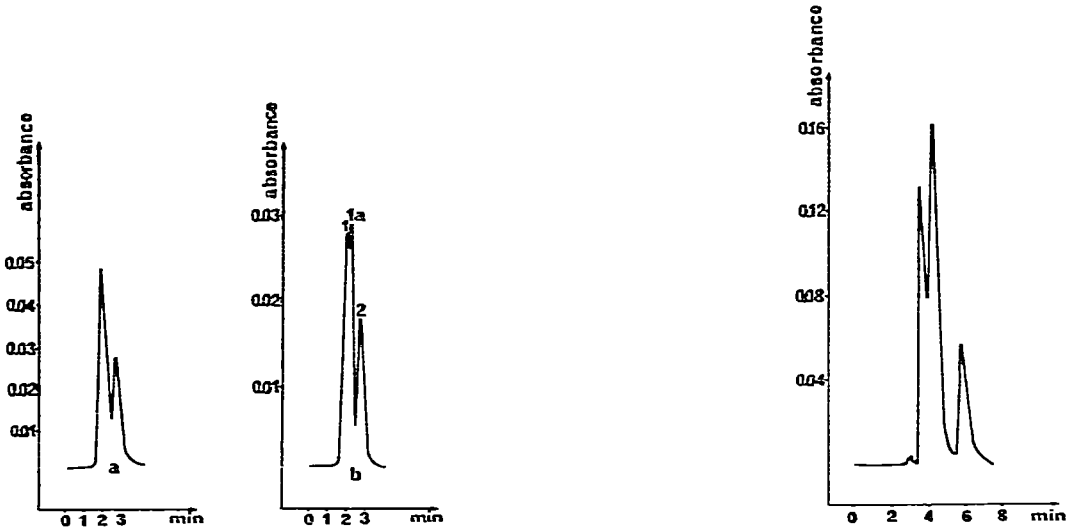

Fig. 1. Effect of the sample concentration on the elution diagram of the kappa-elastin peptides. Concentration of the samples (mg/ml): (a) 1; (b) 0.0156 (1/64). Experimental conditions: see Table III.

Fig. 2. Elution diagram of the kappa-elastin peptides from the 30-cm column (0.45 cm I.D.). Eluent, ethanol–10^{-2} *M* ammonium acetate (60:40, v/v); injected volume, 5 μl.

stances was demonstrated by electric birefringence[6].

The possibility of the separation of the elastin fraction with the column decreases with increasing ethanol concentration in the eluent (Table II). The difference in the elution times of peaks 1 and 2 decreases from 1.4 to 0.9 min when the alcohol–buffer ratio is altered from 60:40 to 80:20. Alcohol concentrations higher than 70% (v/v) seem to increase the amount of material eluted in peak 2, but separation is less complete under these conditions.

Elution volumes remain constant with the dilution of the injected samples (Table III). A new peak (1a) appears between peaks 1 and 2, when highly diluted samples (from 1/32 of the initial concentration, 1 mg/ml) are injected, even on a short (7.5 cm) column (Fig. 1). It seems that high dilution of the injected sample increases the dissociation of the different interacting peptides and allows better separation.

With 30-cm columns with the same stationary phase and the ethanol–ammonium acetate (60:40) solution as the eluent, the elastin peptide mixture was separated in three fractions, even for samples of 1 mg/ml concentration (Fig. 2). Thus the preparative separation of the three fractions was achieved with this eluent system. Upon eluting this column with aqueous solution alone, or with a 90:10 ethanol–aqueous solution, only two peaks appear.

Amino-acid analysis of the separated peak materials (Table IV) indicates that the individual peaks represent different polypeptide populations. Alkaline degradation of fibrous elastin under the conditions used[2,3] yields a large number of homologous peptides. Thus the three main peaks obtained cannot represent homogeneous substances, but it can be assumed that different polypeptide populations[3] are enriched in each of the peaks. Peaks 1 and 2 contain alanine- and glycine-rich peptides, respectively. Such peptide populations were isolated also by isoelectric focusing from

TABLE IV

AMINO ACID COMPOSITION OF THE SEPARATED PEPTIDES

Values are residues per 100 residues.

Peak 1	*Peak 1a*	*Peak 2*	*Amino acid*
1.6	1.9	1	Hyp
1.5	0.7	2.6	Asp
2.5	0.6	1.3	Trp
1.4	0.5	1.9	Ser
4.2	1.7	4.5	Gly
9.8	13.6	10.3	Pro
21.3	27.9	26.28	Gly
39.6	19.7	32.05	Ala
10.1	25.8	10.26	Val
1.5	1.3	1.3	Ile
3.8	4.0	4.5	Leu
0.5	0.4	0.5	Tyr
2.5	1.4	3.2	Phe
0.2	0.3	0.6	Ids
0.3	0.4	0.6	Des
0.2	0.3	0.4	Lys
Trace	Trace	0.1	Arg

the low molecular weight kappa-elastin preparations[3]. Valine-rich peptides enriched in the peak 1a could not been isolated from those preparations in the isoelectric focusing systems already described[3]. Valine-rich peptides were isolated from proteolytic digests of tropoelastine, the precursor of the fibrous elastin[7,8]. These peptides containing repetitive penta- or hexapeptides sequences are distant from the cross-linking regions, and the main structural element is the β-sheet conformation[9]. It has been suggested that this molecular arrangement is a requirement for the elastomeric properties of the elastic fibres[10].

It appears that HPLC on diol-bonded silica supports is suitable for the separation, assay, and study of the aggregation of the main peptide population of alcohol-solubilized elastin peptides. As these different peptides are derived from different regions of the native elastin molecule[3,8], the study of their ratio may be useful in the comparison of elastin preparations originating from normal and pathological tissues.

REFERENCES

1 S. M. Partridge, H. F. Davies and G. S. Adair, *Biochem. J.*, 61 (1955) 11.
2 L. Robert and N. Poullain, *Bull. Soc. Chim. Biol.*, 45 (1963) 1317.
3 M. Moczar, E. Moczar and L. Robert, *Connective Tissue Res.*, 6 (1979) 207–213.
4 A. I. Lansing, T. B. Rosenthal and A. M. Dempsey, *Anal. Rec.*, (1952) 114–555.
5 M. Anselme and B. Sebille, *Anal. Biochem.*, in press.
6 M. Moczar and D. Bernango, unpublished results.
7 W. R. Gray, L. B. Sandberg and J. A. Foster, *Nature* (1973) 246–461.
8 J. A. Foster, E. Bruenger, W. R. Gray and L. B. Sandberg, *J. Biol. Chem.*, 248 (1973) 2876.
9 D. W. Urry, K. Okamoto, R. D. Harris, C. F. Hendrix and M. M. Long, *Biochemistry*, 15 (1976) 4803–4089.
10 D. W. Urry, M. Mammi and L. Gotte, *Bull. Mol. Biol. Med.*, 2 (1977) 157–164.