Journal of Chromatography, 487 (1989) 41–50
Biomedical Applications
Elsevier Science Publishers B V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4490

REVERSED-PHASE LIQUID CHROMATOGRAPHY OF ELASTIN PEPTIDES

JOHN N. MANNING, GREGORY S. SULLIVAN and PAUL F. DAVIS*

The Malaghan Institute of Medical Research, Wellington School of Medicine, Wellington Hospital, Wellington (New Zealand)

(First received July 20th, 1988; revised manuscript received September 8th, 1988)

SUMMARY

Soluble fragments of elastin are frequently present in biological tissue in small amounts. Because of their hydrophobic character, these peptides are not well resolved by a number of conventional techniques. However, their separation should be possible by reversed-phase chromatography. A wide range of columns, gradients and solvents were evaluated. Two systems are described. One was a C_{18} liganded silica column eluted isocratically by gravity flow. Some degree of size fractionation was achieved with larger peptides being eluted with methanol and smaller ones with isopropanol. The second system uses a pressurized elution from another C_{18} ligand column. A concave gradient of trifluoroacetic acid–acetonitrile with a decreasing acetonitrile concentration was optimal. Similar resolution of peptides produced by a variety of digestion methods was obtained with the lower-molecular-mass peptides eluting in the middle of the gradient.

INTRODUCTION

Elastin is regarded as one of the most stable mammalian proteins. Its turnover rate is estimated to be approximately fourteen years in human aorta [1]. Consequently the study of fragments produced in vivo has been difficult. The majority of tissue elastolysis probably results from enzymatic digestion. There are several elastases of different origins and specificities that are candidates as causative agents [2,3]. Isolated preparations of these enzymes have been used to produce peptides for the laboratory study of the degradation [4]. Chemical methods using oxalic acid and alcoholic hydroxide have been utilized to produce soluble peptides [5,6].

It would be desirable to isolate and characterize elastin peptides from tissues in order to understand the hydrolytic mechanisms. In some instances, such as emphysema, this process has been described in some detail [7]; in other cases, such as aneurysms, there is evidence suggesting a possible mechanism [8]. How-

0378-4347/89/$03.50

ever, the degradative aspect of physiological elastin turnover as well as other pathological elastolytic mechanisms are poorly characterized.

The aim of this study has been to develop peptide separation methods that are generally applicable to elastin peptides independent of their origin. To be worthwhile such separations need to provide good resolution no matter what digestion technique is applied. Hence the mechanisms that might be responsible for an observed elastolysis might be determined and evaluated by the use of appropriate marker peptides.

EXPERIMENTAL

Materials

Bovine ligamentum nuchae elastin was obtained from Sigma (St. Louis, MO, U.S.A.). Porcine pancreatic elastase was purchased from Millipore and human leukocyte elastase from Elastin Products (Pacific, MO, U.S.A.). The Sep-Pak and Rad-Pak reversed-phase columns were from Waters Assoc. (Bedford, MA, U.S.A.). The C_3 column (RPSC Ultrapore) (7.5 cm×4.6 mm) was from Beckman (Berkeley, CA, U.S.A.) and the C_8 column (BU-300 Aquapore) (25 cm×4.6 mm) was from Brownlee Labs. (Santa Clara, CA, U.S.A.). The chemicals used in this study were from commercial sources and were of the highest analytical quality (HPLC grade was used whenever possible). All solvents used in chromatography were prepared with Milli-Q water. The scintillation cocktail, ACS-II, and [^{3}H]sodium borohydride (256 mCi/mg) were purchased from the Radiochemical Centre (Amersham, U.K.).

Methods

The reduction of elastin with [^{3}H]sodium borohydride was performed as previously described [3]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble elastin was an adaptation of the Weber–Osborne method. The modification for soluble elastin has been reported previously [9].

Digestion of elastin

Digestion of elastin with oxalic acid was performed according to the method of Partridge et al. [5]. The digestion was stopped by neutralization of the acid with sodium hydroxide. This produced α,β-elastin. A portion of the digest was dialysed against four changes of distilled water over six days to produce α-elastin. Both the original and the dialysed digests were dried.

Elastin was digested with a solution of 1 *M* potassium hydroxide in 80% ethanol at 37° for 5 h [6]. The supernatant was separated following centrifugation and neutralised with acetic acid. This is known as κ-elastin. Some of the supernatant was dialysed against distilled water as described above.

For enzymic digestions, elastin (1 g) was suspended in 15 ml of 0.05 *M* borate buffer pH 8.5. Leukocyte elastase (500 μg, 435 U) in 0.02 *M* ammonium bicarbonate was added and the digest shaken at 37°C for varying times. The hydrolysis was stopped by placing the supernatant in boiling water for 5 min. Digestion with pancreatic elastase (64 U/mg) was performed similarly with the exception that

several different ratios of enzyme to substrate were used. For the material used in Sep-Pak chromatography the digestions were performed at an enzyme/protein ratio of either 1:1000 for 24 h (extensive digestion) or 1:10 000 for 1 h (mild digestion). For the peptides used in HPLC the ratio was also 1:10 000.

Sep-Pak chromatography

The peptide mixture was usually dissolved in 10% of methanol at about 1 mg/ml. An aliquot was loaded on the column and allowed to equilibrate for 45–60 min at room temperature before elution was commenced. A range of solvents was used. In most cases, 4 ml of each solvent were passed through the column and the eluate was collected.

High-performance liquid chromatography

HPLC was undertaken using two Waters Model 510 pumps controlled by a Waters Model 680 automated gradient controller. Detection was carried out with a Waters Model 441 absorbance monitor. A guard column and prefilter were inserted in the line prior to the reversed-phase column. The sample was dissolved in 10% acetonitrile at 2 mg/ml and 25 μl were injected. The flow-rate was 1 ml/min. A number of different eluting solvents and gradients were evaluated. Fractions were collected at 1-min intervals.

Analytical methods

For the estimation of protein, aliquots were removed from the column fractions, dried and hydrolysed in 50% sodium hydroxide for 20 min at 1300 KPa. After cooling, an equal volume of acetic acid was added and then ninhydrin reagent [10] was added and the tubes were placed in boiling water for 15 min. After cooling, 50% ethanol was added and the absorbance was measured at 570 nm.

For amino acid analysis, samples were hydrolysed in vacuo in constant-boiling hydrochloric acid at 110°C for 65 h. The analyses were conducted on a Beckman 119CL amino acid analyser.

For radioactivity measurement, aliquots were removed and added to ACS II scintillation fluid. Scintillation counting was performed in a Philips 4700 instrument.

RESULTS

α-Elastin was chromatographed under a variety of reversed-phase chromatography conditions. These included columns containing C_3, C_8 and C_{18} ligands attached to silica particles, as well as a range of elution conditions in terms of types of solvents, concentrations and sequence of application and temperature. The solvents included dimethylformamide, methanol, isopropanol, acetonitrile, sodium chloride, sodium phosphate, urea, propionic acid, trifluoroacetic acid (TFA), sodium hydroxide and water. The eluates were monitored by ultraviolet detection and fractions that appeared to contain peptides were hydrolysed and analysed either on the amino acid analyser or by a colorimetric determination with ninhydrin.

Resulting from this preliminary survey, two protocols provided considerable resolution of the polypeptides. These are described below.

Sep-Pak chromatography

Reversed-phase chromatography on Sep-Pak columns using step gradients provided resolution of soluble peptides produced from tritium-labeled elastin. Elastin was solubilized by oxalic acid hydrolysis and by digestion with pancreatic elastase (see Experimental).

The peptide pattern obtained by the mild hydrolysis conditions is shown in Fig. 1. There are peptides present between the 36 000 and 55 000 relative molecular mass (M_r) markers and also peptides running faster than the 20 000 M_r marker. Positive staining material is visible at the electrophoresis front. The peptides produced by the more extensive degradation did not stain on the SDS-PAGE system (data not shown).

Table I shows that most of the protein applied to the column from the α-elastin digest was retained and eluted with 50% isopropanol–0.2% TFA. The elution profile for radioactivity indicates that approximately 27% of the applied tritium eluted with isopropanol.

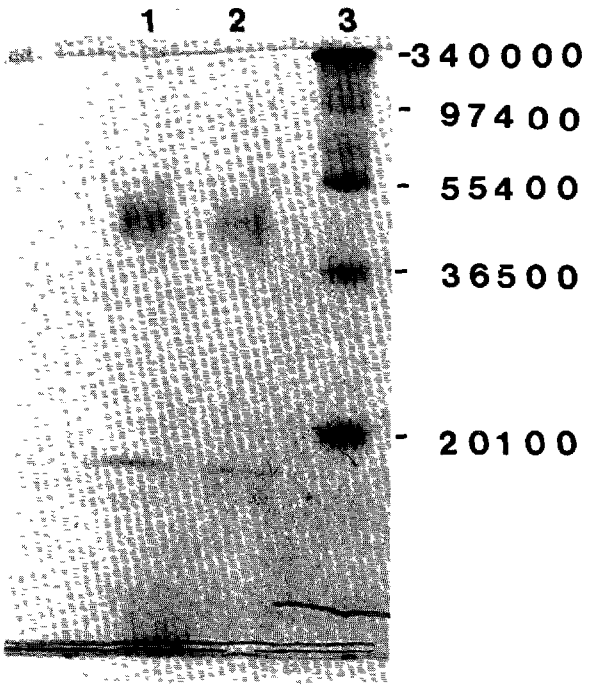

Fig. 1. SDS-PAGE of soluble products arising from the digestion of elastin by pancreatic elastase at a ratio of 10 000:1 (w/w) for 1 h at 20 °C in 0.2 *M* sodium chloride in 0.02 *M* phosphate pH 7.3. The electrophoresis and staining conditions are described in Experimental. Lane 1, 20 μl of sample; lane 2, 10 μl of sample; lane 3, M_r markers (α_2-macroglobulin, M_r=340 000; phosphorylase b, M_r=97 400; glutamate dehydrogenase, M_r=55 400; lactate dehydrogenase, M_r=36 500; trypsin inhibitor, M_r=20 100).

TABLE I

ELUTION OF ELASTIN PEPTIDES FROM A SEP-PAK REVERSED-PHASE COLUMN

Eluent	α-Elastin*		Mild elastase digestion**		Extensive elastase digestion***	
	Protein (%)	Tritium (%)	Protein (%)	Tritium (%)	Protein (%)	Tritium (%)
10% Methanol	8.7	56.8	68.5	61.5	12.7	39.6
0.2 *M* Sodium chloride	15.9	8.1	13.9	4.0	2.8	0.7
50% Isopropanol	75.4	26.8	16.4	25.5	84.5	39 5

*α-Elastin was prepared according to the method of Partridge et al. [5].

**Elastin was digested with pancreatic elastase at an enzyme/protein ratio of 1·10 000 for 1 h at 37°C.

***Elastin was digested with pancreatic elastase at an enzyme/protein ratio of 1·10 000 for 24 h at 37°C.

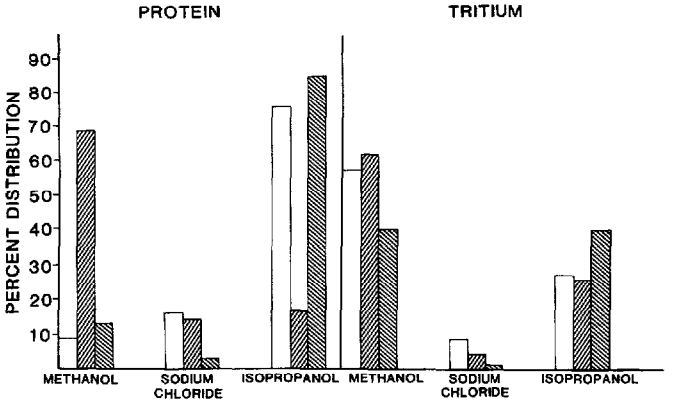

Fig. 2. Distribution of protein and tritium during chromatography of solubilized elastin on Sep-Pak columns. (□) α-Elastin; (▨) digestion of elastin with pancreatic elastase (10 000.1) for 1 h at 20°C; (▧) digestion of elastin with pancreatic elastase (1000:1) for 24 h at 20°C.

TABLE II

COMPOSITION OF FRACTIONS ELUTED FROM THE SEP-PAK COLUMN

Amino acid	Composition (residues/1000 residues)					
	Mild elastase digestion*			Extensive elastase digestion**		
	Methanol	Sodium chloride	Isopropanol	Methanol	Sodium chloride	Isopropanol
Glycine	389.0	142.9	177.8	233.4	265.3	381.4
Alanine	89.6	49.4	73.7	378.5	66.3	194.5
Hydroxyproline	71.9	–	–	–	–	6.5
Valine	24.7	39.5	71.6	94.4	209.0	154.4

*Elastin was digested with pancreatic elastase at an enzyme/protein ratio of 1:10 000 for 1 h at 37°C.

**Elastin was digested with pancreatic elastase at an enzyme/protein ratio of 1:10 000 for 24 h at 37°C.

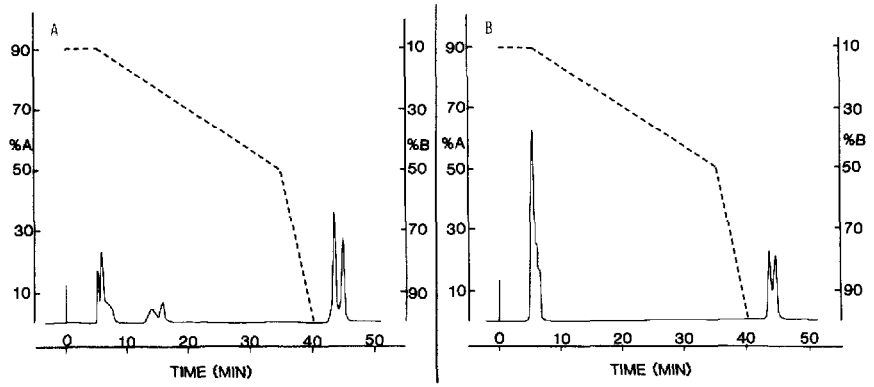

Fig. 3. Chromatography on a Rad-Pak C_{18} reversed-phase column of elastin peptides produced by oxalic acid digestion of bovine ligamentum nuchae elastin using different solvents. The flow-rate is 1 ml/min. (A) solvent A is 0.1% trifluoroacetic acid; solvent B is 100% acetonitrile. (B) solvent A is 0.01 *M* ammonium bicarbonate; solvent B is 100% acetonitrile.

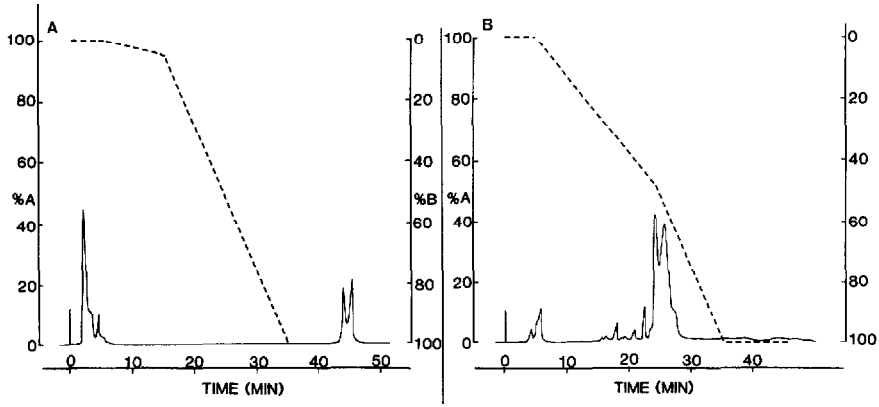

Fig. 4. Chromatography on a Rad-Pak C_{18} reversed-phase column of elastin peptides produced by pancreatic elastase digestion of bovine ligamentum nuchae elastin. The flow-rate is 1 ml/min. (A) Solvent A is water, solvent B is 100% acetonitrile. (B) Solvent A is 100% acetonitrile; solvent B is water.

The elution profile for the peptides produced by mild digestion with elastase shows that the majority of the protein eluted with 10% methanol. However, with more extensive digestion only a small proportion of the soluble peptides were present in this solvent. The majority was bound to the column and eluted with the isopropanol–TFA (Table I and Fig. 2).

The majority of the tritium label was also not retained by the column when the peptides from the mild digest were chromatographed. Interestingly, the specific radioactivity was higher in the isopropanol eluate. For the more extensive digest there was approximately equal radioactivity in both alcoholic eluates but because of the reduced protein content the specific radioactivity is much higher in the methanol eluate (Table I and Fig. 2).

Amino acid analysis of the fractions from the chromatography of the peptides produce by mild digestion showed that both the glycine and hydroxyproline con-

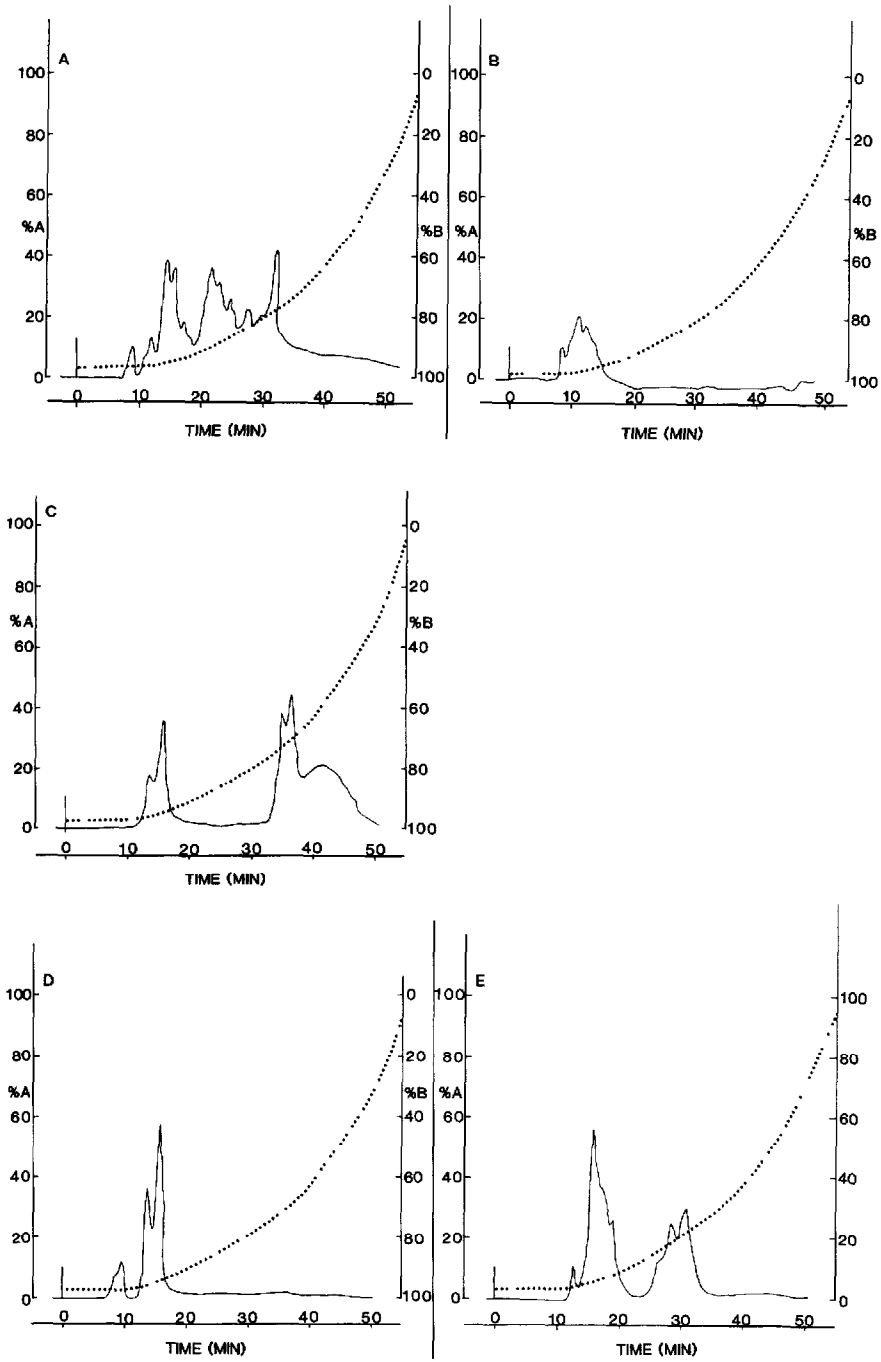

Fig. 5. Chromatography on a Rad-Pak C_{18} reversed-phase column of elastin peptides produced by digestion of bovine ligamentum nuchae. The flow-rate is 1 ml/min. Solvent A is 0.05% trifluoroacetic acid; solvent B is 100% acetonitrile. (A) Oxalic acid digest (α,β-elastin). (B) Non-dialyzable fraction of α,β-elastin (α-elastin). (C) Pancreatic elastase digest (85% solubilization). (D) Leukocyte elastase digest (35% solubilization). (E) Leukocyte elastase digest (57% solubilization).

tent were highest in the methanol and that valine was highest in the isopropanol eluate. The alanine content was less than anticipated in all three solvents (Table II). In contrast, the highest glycine proportion for the extensively digested elastin fragments was found in the isopropanol fraction. There were also elastin-like levels of hydroxyproline, alanine and valine in this fraction.

C_{18} μBondapak HPLC

Several types of reversed-phase HPLC columns were tested for the resolution of peptides produced by digestion of insoluble elastin. These included C_3 and C_8 aliphatic chains linked to silica. The best, however, was the C_{18} reversed-phase column.

Following the establishment of the most appropriate chromatographic matrix, the investigation of the most suitable solvent system was undertaken. Numerous solutions and programmes were tested using a preparation of α,β-elastin. These included buffers (acidic, neutral and basic), salt gradients, alcohols and ion-pairing reagents. Examples of several of these are shown in Fig. 3. The best separation was achieved with a non-linear gradient of acetonitrile and 0.05% TFA.

The samples were initially chromatographed with a gradient of increasing acetonitrile concentration. The majority of the peptides eluted at less than 10% of acetonitrile (Fig. 4A). Using a shallower gradient at low acetonitrile concentrations did not improve the resolution. However, if the gradient was reversed and the peptides were eluted with a decreasing concentration of acetonitrile, the fractionation was improved (Fig. 4B). Chromatography using such a gradient programme showed good resolution for mixtures of elastin peptides produced by several different methods of digestion. These include oxalic acid (Fig. 5A and B), pancreatic elastase (Fig. 5C) and leukocyte elastase (Fig. 5D and E).

Preliminary analyses of the major fractions suggest that the peptides that elute early (at relatively high acetonitrile concentrations) have a relatively high content of hydrophilic amino acids. In particular the ratio of acidic amino acids relative to glycine is greater than in the later eluting fractions.

DISCUSSION

Elastin is normally a very stable molecule with a long half-life [1]. In some pathological conditions, such as lung emphysema, aortic aneurysms and atherosclerosis, there is an accelerated rate of loss [7,8,11]. In these conditions the sites of cleavage in the elastin molecule are largely unknown. One approach to clarifying this is to compare the structure of the hydrolytic products with the parent fibrous molecule. In order to do this, the soluble peptides need to be fractionated. Because of the hydrophobic nature of elastin, many of the conventional methods of peptide separation are likely to be unsuccessful. These include electrophoresis, ion-exchange chromatography and isoelectric focussing. Amongst the few methods of separation of elastin fragments reported previously have been gel permeation chromatography [12], hydrophobic interaction chromatography [9,13] and C_{18} reversed-phase chromatography [14].

In this report two fractionation procedures using reversed-phase chromato-

graphy are reported. They are suitable for peptides produced by several digestion procedures and so are probably applicable to the characterization of elastin peptides produced in tissue by unknown mechanisms. Some insight into the elution behaviour of the large and small peptides has been gained.

The use of the Sep-Pak columns enabled a variety of eluents to be evaluated for the separation of elastic peptides by reversed-phase chromatography. Methanol and isopropanol were the best solvents tested. The more polar solvents eluted relatively little of the digest as judged either by ninhydrin analysis or by radioactivity determination. Some fractionation can also be achieved from the use of the different-chain-length alcohols. With mild digestion methods, there should be a higher proportion of larger peptides. SDS-PAGE of this digest indicates peptides near the 20 000 M_r marker and between the 36 000 and 55 000 M_r markers. In these chromatography conditions most of the peptide and radioactivity is present in the methanol fraction, and the glycine content is highest in this eluate. When the number of smaller peptides increases, there is an increase in the protein and tritium content in the isopropanol fraction. This eluate is very elastin-like with respect to the glycine, alanine, valine and hydroxyproline content.

Separation of mixtures of elastin peptides by HPLC has been difficult because of their hydrophobic nature and size which often leads to retention on the columns. Some successful separations have been performed but these were only carried out with peptides produced by alcoholic potassium hydroxide digestion [14]. Oxalic acid digestion is rather non-specific producing a wide range of peptides. Resolution of this material on the C_{18} μBondapak column was performed with an acetonitrile–water gradient. Substituting 0.01 *M* ammonium bicarbonate for water provided some improvement but 0.05% TFA was better. In all cases though, retention was high. A dramatic change was obtained by running a reverse gradient, i.e. decreasing acetonitrile concentration. Such a programme was also successful with the alcoholic potassium hydroxide and the enzymatic digests.

When the lower-molecular-mass peptides are removed from the α,β-elastin, the peptides eluting between approximately 7 and 20% acetonitrile disappear. These are the smaller fragments. Consistent with this is the observation that there is an increase in peptides eluting in this region in the extensively digested material resulting from elastase incubation. However, in all of these digests there is material that is not retained on the column and elutes early.

This paper shows that there are at least two systems for the fractionation of elastin peptides which have been produced by a variety of hydrolysis techniques. It is possible to combine the procedures with the Sep-Pak columns providing an initial separation and to resolve the resulting fractions further by HPLC. It seems possible that soluble fragments of elastin produced by uncharacterized means could also be resolved by reversed-phase chromatography.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance received from the Wellington Medical Research Foundation and the Medical Research Council of New Zealand for this project.

REFERENCES

1 R.B. Rucker and D. Tinker, Int. Rev. Exp. Pathol., 17 (1977) 1.
2 L.B. Sandberg, N.T. Soskel and J.G. Leslie, N. Engl. J. Med., 304 (1981) 506.
3 P.J. Stone, C. Franzblau and H.M. Kagan, Methods Enzymol., 82 (1982) 588.
4 R.M. Senior, D.R. Bielefeld and B.C. Starcher, Adv. Exp. Med. Biol., 79 (1977) 249.
5 S.M. Partridge, H.F. Davis and G.S. Adair, Biochem. J., 61 (1955) 11.
6 M Moczar, E. Moczar and L. Robert, Connect. Tissue Res., 6 (1979) 207.
7 A. Janoff, Am. Rev. Respir. Dis., 132 (1985) 417.
8 J.S. Campa, R.M. Greenhalgh and J.T. Powell, Atherosclerosis, 65 (1987) 13.
9 P.A. Ryan, J.N. Manning and P.F. Davis, J. Chromatogr., 275 (1983) 31.
10 W.H. Stein, J. Biol. Chem., 211 (1954) 907.
11 L. Robert and A.M. Robert, in A.M. Robert and L. Robert (Editors), Frontriers of Matrix Biology, Vol. 8, Karger, Basel, 1980, p. 130.
12 E.G. Clearly and W.J. Cliff, Exp. Mol. Pathol., 28 (1978) 227.
13 P.F. Davis, P.A. Ryan, J.N. Manning, W.E. Stehbens and S.J.M. Skinner, Angiology, 35 (1984) 38.
14 B.P.D. Tuy and E. Moczar, J. Chromatogr., 291 (1984) 445.