Journal of Chromatography, 272 (1983) 366—372
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1497

Note

Reversed-phase high-performance liquid chromatography method for separation of collagen tryptic peptides

ALBERT J. BANES* and MITSUO YAMAUCHI

Department of Surgery and Dental Research Center, University of North Carolina, Chapel Hill, NC 27514 (U.S.A.)

and

ROBERT T. MOODY

E.I. du Pont de Nemours & Co., Analytical Instruments Division, Wilmington, DE 19898 (U.S.A.)

(First received July 19th, 1982; revised manuscript received September 9th, 1982)

Recent methods developed for use with high-performance liquid chromatography (HPLC) for the separation of large peptides or proteins include gel chromatography with Toyo Soda TSK columns or Waters I series columns in 6 *M* guanidine HCl, 1 *M* Tris buffer or 0.1 *M* potassium phosphate, pH 7.0, and reversed-phase chromatography on octadecylsilane (C18) or cyanopropyl (CN) columns with a variety of acid aqueous—organic eluents [1—7]. In particular, collagen alpha chains (approximately 93,000 daltons) from Types I, II and III collagens have been resolved on cyanopropyl columns using a pyridine—acetate—propanol gradient [8]. Cyanogen bromide peptides of collagen have also been separated on reversed-phase columns [7]. However, separation and identification of tryptic peptides of collagen are still performed principally using gel electrophoresis or low-pressure column chromatography [9—11]. The current investigation presents a method for the isocratic separation of collagen tryptic peptides on large pore size, octadecylsilane, reversed-phase columns.

MATERIALS AND METHODS

Columns

The reversed-phase column used in these experiments was a DuPont Zorbax

0378-4347/83/0000—0000/$03.00

octadecylsilane (C18, ODS), 25 cm × 4.6 mm, with 5 μm particle size and 150 Å pore size (DuPont prototype column, 25-85-3).

Sample preparation

Bone diaphyses from four-week-old osteoblastoma chicks were placed on ice and stripped of periosteum and marrow, cut into 5-mm^3 pieces with bone rongeurs and pulverized to 200—400 mesh powder (at temperature of liquid nitrogen) using a Spex freezer mill (Spex, Metuchen, NJ, U.S.A.). Bone powder was demineralized in repeated changes of 0.5 *M* EDTA, 0.05 *M* Tris buffer pH 7.4 at 4°C, the residue washed exhaustively in 0.02 *M* NH_4HCO_3 and water and the residue lyophilized and weighed. A portion of the residue (largely bone collagen) was suspended in 10 m*M* calcium chloride in 50 m*M* Tris buffer pH 7.5, heated at 65°C for 20 min, cooled to 37°C, then mixed with trypsin (1% w/w, TPCK trypsin, cat. No. 30C637, Millipore, Freehold, NJ, U.S.A.) and incubated at 37°C for 2 h with stirring [10, 11]. A second trypsin portion (half of the starting amount) was added after 2 h and hydrolysis continued for two more hours [10, 11]. After hydrolysis, the residue was lyophilized, weighed, and a portion dissolved at 37°C for 20 min in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 (guanidine HCl obtained from Research Plus Laboratories, Denville, NJ, U.S.A.). Samples were sedimented at 1000 *g* for 5 min; then the supernatant fluid was removed for chromatography.

Liquid chromatography systems

Three separate chromatography systems were employed: (1) the DuPont Model 8800 controller with the Model 870 triple head pump, single sample injector, column heater, variable-wavelength detector (set at 214 nm, 8-μl flow cell) and recorder; (2) a Beckman 110 pump, single sample injector, Hitachi Model 110-10 variable-wavelength detector (set at 214 nm, 20-μl flow cell) and recorder; (3) a Waters Assoc. system controller, WISP 710 B, multisample injector, two Model 6000A pumps, a Model 441 absorbance detector (214 nm, 12-μl flow cell) and a data module.

In some experiments, sample fluorescence was monitored using a Perkin-Elmer fluorometer (Model 204-A, excitation 325 ± 5 nm, emission 395 ± 5 nm). Selected peptides were collected from five individual separations, dialyzed against water, lyophilized, and subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS) acrylamide gels.

CHROMATOGRAPHY

Mobile phase solvents were filtered through a 0.22-μm filter and degassed prior to use.

Low-pressure chromatography

For chromatography by conventional means, samples (120 mg) were applied to 300 cm × 1.5 cm columns of Sephadex G-50 superfine gel in 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.5 at a flow-rate of 15—20 ml/h.

Isocratic HPLC

Samples were applied to the column in mobile phase and chromatographed at 35°C in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 at a flow-rate of 1 ml/min (pressure 40 bar). In other experiments, concentrations of guanidine HCl up to 2 *M* were used, the pH was varied from 6.5 to 7.5, the temperature varied from 22°C to 60°C, and the flow-rate altered from 0.3 ml/min to 2.0 ml/min.

HPLC gradients

Linear gradients (60 ml) consisting of 1—500 m*M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 at 1 ml/min and more shallow gradients were utilized. Moreover, gradients of guanidine HCl and methanol were also utilized.

RESULTS

Molecular weight determination of the tryptic peptides of collagen in 1 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 on a TSK-2000 column and two I-60 columns indicate a range of 10,000—20,000.

Fig. 1 depicts a chromatogram of 120 mg of bone collagen on a G-50 superfine gel eluted with 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.4 monitored at 230 nm. A large front peak (1) containing highly crosslinked collagen

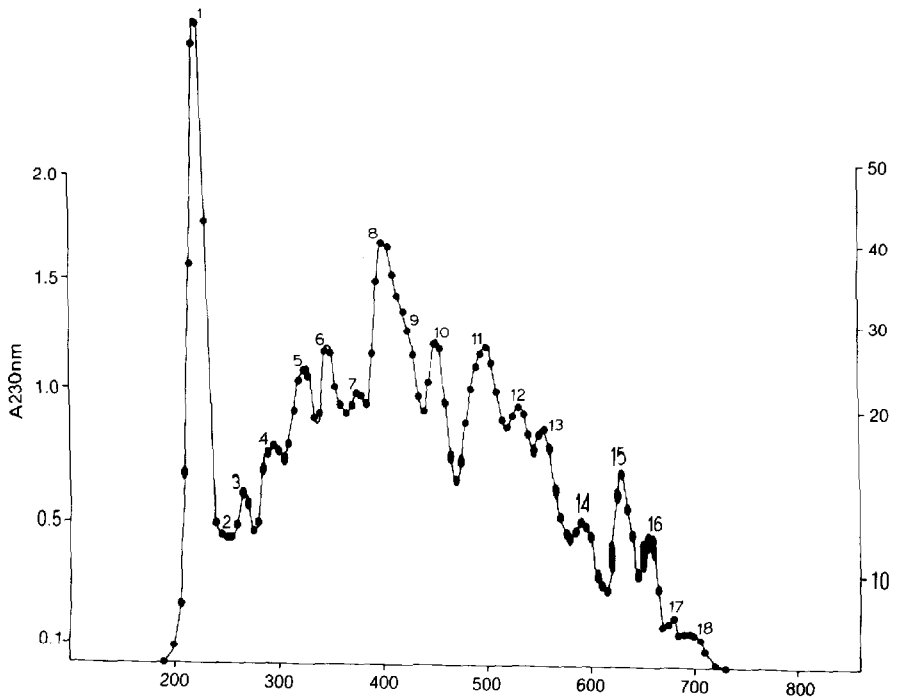

Fig. 1. Chromatogram of 120 mg of bone collagen (not reduced with $NaBH_4$) tryptic peptides from an osteoblastoma from a three-month-old chicken dissolved in 3.0 ml of 5 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.2 at 22°C. The sample was separated on a 300 × 1.5 cm column of G-50 superfine gel in 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.5 at a flow-rate of 15—20 ml/h. Fractions of 5 ml were collected and monitored at 230 nm. Eighteen peaks were resolved using this method; peaks 1 (void volume peak) and 3 were heterogeneous after isolation and separation on acrylamide gels.

peptides elutes in the void volume. At least 17 other peaks were detected; peaks 1 and 3 were heterogeneous by SDS gel electrophoresis (Fig. 1). In particular, peak 3, a fraction containing the fluorescent crosslink 3-hydroxypyridinium, is comprised of at least three peptides [12].

Fig. 2 is a chromatogram, typical of separations on the Zorbax C18 column at 35°C in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 with 100 μg of sample applied. However, less than 10 μg of sample can be chromatographed containing at least 30 peaks ($A_{214\ nm}$). Three of these peaks (peaks 1, 21 and 25 in Fig. 2) exhibited fluorescence (excitation = 325 nm, emission = 395 nm), indicating presence of 3-hydroxypyridinium [11]. Identical peaks (peak 21, Fig. 2) from five separations (1 mg sample size) were collected, dialyzed against water, lyophilized, and electrophoresed on 10% SDS gels. Each gave a single, faint, Coomassie-Blue reactive band in an acrylamide gel (data not shown).

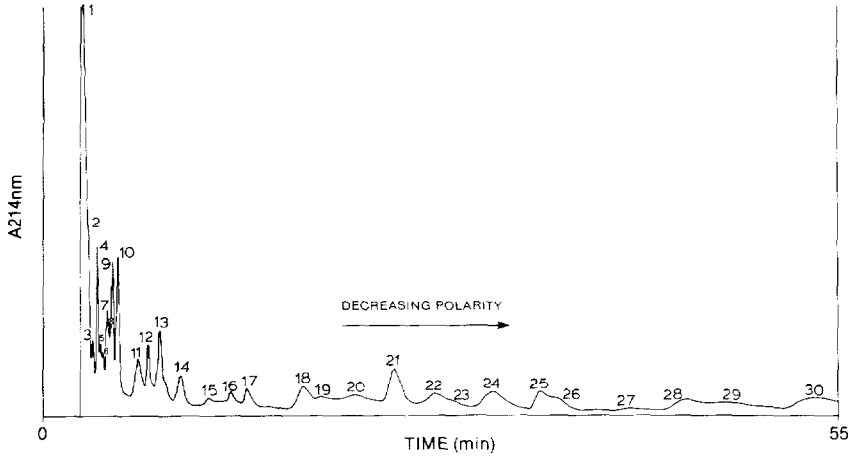

Fig. 2. Chromatogram of 100 μg of bone collagen tryptic peptides from an osteoblastoma from a three-month-old chicken dissolved in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8. The sample was injected onto a 25 cm × 4.6 mm octadecylsilane (C18) column, 5 μm particle size, 150 Å pore size (prototype DuPont column) and eluted at 1 ml/min at 35°C with mobile phase as above. Thirty distinct peaks were resolved, three of which (peaks 1, 21 and 25) fluoresced when analyzed with a Perkin-Elmer fluorometer at excitation 325 nm and emission 395 nm. In some experiments, a dye-binding assay (Bio-Rad) was used to monitor peptides in column fractions. Peak 21 was collected, concentrated and electrophoresed on a 10% SDS gel. A single Coomassie-Blue staining band was visualized.

The baseline returned to zero after 2 h for most collagen tryptic peptide samples tested and no peaks were detected during 18 h of additional run time. Series injections of the same sample yielded excellent reproducibility in peak retention times and shape.

The chromatogram in Fig. 3 depicts separation at room temperature (22°C) in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8. At least 47 peaks were detected ($A_{214\ nm}$).

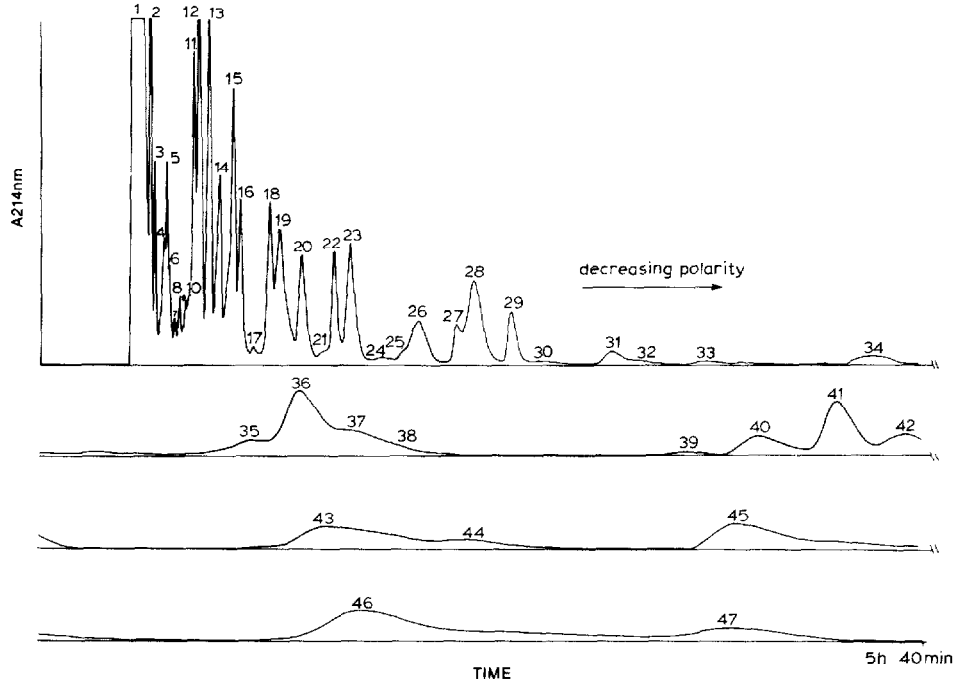

Fig. 3. Chromatogram of 50 μg of bone collagen tryptic peptides from an osteoblastoma from a three-month-old chicken dissolved in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8. The sample was injected onto a 25 cm × 4.6 mm octadecylsilane (C18) column, 5 μm particle size, 150 Å pore size (DuPont prototype column), and eluted at 1 ml/min at 22°C with mobile phase as above. Forty-seven distinct peaks were resolved. The total run time was 5 h 40 min. In some experiments a dye-binding assay (Bio-Rad) was used to monitor peptides in column fractions.

DISCUSSION

Reversed-phase chromatography is a reliable method to resolve tryptic peptides of collagen. Moreover, the latter technique permits greater resolution, reduces run time (1—2.5 h per sample) and is useful for microgram quantities of peptides. By comparison, molecular sieve-type gels offer less resolution in a single separation of a complex peptide mixture, require longer run times (days) and usually require milligram quantities of sample. The advantage in the latter case is the analysis of larger sample sizes, useful when preparing large quantities of a single peptide.

In addition, state-of-the-art protein separation columns such as the Toyo Soda (TSK 2000 or 3000, Toyo Soda Manufacturing Co., Tokyo, Japan) or Waters I-60 series columns with 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.4, used with HPLC, did not yield sufficient resolution of collagen tryptic peptides compared with that of reversed-phase columns (patterns were similar to that in Fig. 1 for samples separated on one TSK 2000 and two I-60 columns joined in tandem).

Isocratic separations in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 at 35°C, 1 ml/min yielded the most satusfactory results (Fig. 2). These con-

ditions yielded good sample solvation, resolution and separation of collagen tryptic peptides in 1—2 h for most samples. The room temperature separation (Fig. 3) yielded more peaks (47) than did that at 35°C (30 peaks) but required 4 h and 40 min longer to effect the chromatography. Increasing the molarity of guanidine HCl up to 2 *M* decreased retention time to approximately 20 min, but yielded fewer peaks with reduced resolution, particularly in early eluting peaks. Solvent strengths above 0.5 *M* guanidine HCl were unsatisfactory, 0.1 *M* to 0.5 *M* yielded poor results, and those below 0.1 *M* yielded good to excellent results. The influence of pH in the narrow range of 6.5—7.5 had no influence on resolution (data not shown). Increased temperature decreased retention times, but also reduced the total number of peaks eluting (data not shown). The range of 35—41°C yielded similar and satisfactory results at the 0.05 *M* guanidine HCl concentration, whereas 45—60°C yielded increasingly poor results.

Programmed flow-rate increases were useful in reducing total chromatography time in the isocratic mode. For instance, a flow-rate of 0.5 ml/min was used for 10 min to increase separation of early eluting peaks, was increased to 1 ml/min for 10 min (to a point where peaks 19 and 20 elute, Fig. 2), then was increased to 2.0 ml/min for another 10 min (to the region of peak 26), then up to 3.0 ml/min for 10 min, with a return to initial conditions for 5 min before the next sample application. The latter method can be used with the Waters Assoc. LC system which is flow programmable. Flow-rate is a major consideration with any peptide separation when fraction collection is involved. Peptides separated by a matter of seconds are difficult to collect, therefore reduced chromatography time is not always beneficial. A total run time of 1 h allows for collection of 0.5-ml fractions with, in most cases, one-tube peaks. Use of a peak collector simplifies peptide collection.

Gradient elution with increasing guanidine HCl concentration from 1 m*M* to 500 m*M* was not satisfactory due to baseline shifts at 214 nm. More shallow gradients were tried (50—100 m*M* salt) but baseline shifts proved to be an overwhelming problem. Increasing the wavelength to 230 nm or 280 nm for collagen did not solve the latter problem. Methanol—guanidine HCl gradients gave the same results — unsatisfactory rises in baseline. Most recently, acetate—acetonitrile or trifluoroacetic acid—acetonitrile gradients have been tried, but some samples did not completely dissolve in initial mobile phase and some collagen peptides appeared to bind very strongly to the stationary phase in acetonitrile.

Few reports have been published on the use of halide-containing mobile phases in mildly acidic conditions for peptide elution from gel permeation or reversed-phase columns [6]. Most manufacturers do not recommend the use of halides, especially in acid conditions, in stainless-steel HPLC equipment. However, the pump heads, tubing and columns in the DuPont and Beckman systems have been used for approximately 1 year with either 0.05 *M* or 2 *M* guanidine HCl, respectively, with no demonstrable adverse effects.

The total number of collagen tryptic peptides separated by these systems was 30 at 35°C and 47 at 22°C. The theoretical number of peptides obtainable if trypsin hydrolyzed at every lysine (33 residues) and arginine (53 residues) residue in the alpha 1 chain of Type I collagen would be 86. Some of

the lysine residues are involved in crosslink formation, thus reducing the total number of possible peptides. Although every tryptic fragment may not be resolved, the isocratic method for separation of collagen tryptic peptides reported in the present investigation not only has the best resolution for collagen tryptic peptides reported to date, but also has great flexibility in sample loading. Sample amounts can be applied ranging from micrograms to several milligrams without causing major changes in the elution times. It is possible to apply a 1-mg sample, collect a particular peak, dialyze and lyophilize the sample, and prepare enough of one peptide in a day, to do amino acid and/or sequence analysis. This procedure represents a significant advance in collagen tryptic peptide resolution as well as providing the most rapid method for analysis to date.

ACKNOWLEDGEMENTS

This study was supported by NIH grants DE-02668, RR-05333, and the Department of Surgery, University of North Carolina.

REFERENCES

1 D.R. Burger, A.A. Vandenbark, W. Dunnick, W. Kraybill, G.D. Daves and R.M. Vetto, J. Immunol., 122 (1979) 1091.
2 I.M. Chaiken and C.J. Hough, Anal. Biochem., 107 (1980) 11.
3 A. Fallon, R.V. Lewis and K.D. Gibson, Anal. Biochem., 110 (1981) 318.
4 R.A. Jenik and J.W. Porter, Anal. Biochem., 111 (1981) 184.
5 W.C. Mahoney and M.A. Hermodson, J. Biol. Chem., 255 (1980) 11199.
6 N. Ui, Anal. Biochem., 97 (1979) 65.
7 M. van der Rest, H.P.J. Bennett, S. Solomon and F.H. Glorieux, Biochem. J., 191 (1980) 253.
8 R.V. Lewis, A. Fallon, S. Stein, K.D. Gibson and S. Udenfriend, Anal. Biochem., 104 (1980) 153.
9 P.T. Kelly, K. von der Mark and G.W. Conrad, Anal. Biochem., 112 (1981) 105.
10 Y. Kuboki, M. Tsuzaki, S. Sasaki, C.F. Liu and G.L. Mechanic, Biochem. Biophys. Res. Commun., 102 (1981) 119.
11 M. Yamauchi, A.J. Banes, Y. Kuboki and G.L. Mechanic, Biochem. Biophys. Res. Commun., 102 (1981) 59.
12 M. Yamauchi, A.J. Banes, Y. Kuboki and G.L. Mechanic, in preparation.