Isolation and Characterization of the Cyanogen Bromide Peptides Derived from the Human α2(V) Collagen Chain†

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ABSTRACT: The human α2(V) collagen chain when cleaved with cyanogen bromide yields ten peptides which can be recovered in approximately equimolar quantities. Characterization of the purified peptides with regard to molecular weight and amino acid composition establishes the uniqueness of the peptides and reveals that the α2(V) chain recovered following limited pepsin digestion contains 956 amino acid residues. Possible homologies between the α2(V) peptides and peptides derived from other collagen chains are noted. In addition, a high-performance liquid chromatography system is described for the separation of three of the α2(V) chain peptides which were not resolved by using conventional separation techniques.

Current investigations into the nature and organization of the vertebrate collagens have led to the discovery of several new collagenous components. In addition to the already well-characterized chains of the interstitial type I, II, and III collagens (Miller, 1976) and the more recently described type IV basement membrane components (Kresina & Miller, 1979; Gay & Miller, 1979; Glanville et al., 1979), there exist three chains of the type V collagen group. These are the α1(V) or B chain,[1] the α2(V) or A chain, and the α3(V) or αC chain (Burgeson et al., 1976; Chung et al., 1976; Brown et al., 1978; Sage & Bornstein, 1979). Compositionally, the latter chains exhibit certain features which distinguish them from the interstitial and basement membrane collagen chains. Specifically, these chains have lower quantities of alanine, elevated levels of hydrophobic residues, and increased hydroxylation and glycosylation of lysine compared to the interstitial chains. Comparison with the chains of type IV collagen shows the type V chains to contain proportionately fewer hydrophobic groups with lower levels of hydroxylation of both prolyl and lysyl residues.

Some initial studies on type V indicated that the α1(V) and α2(V) chains were present in a 2:1 ratio, and it was suggested that the molecular configuration for native type V was $[\alpha 1(V)]_2\alpha 2(V)$ (Burgeson et al., 1976; Bentz et al., 1978). Concomitantly, other reports demonstrated that the ratio of the α1(V) and α2(V) chains was a function of the tissue used in the extraction (Rhodes & Miller, 1978; Brown et al., 1978; Sage & Bornstein, 1979). In placenta, the ratio of α1(V) to α2(V) was found to be 1:1, and in articular cartilage α1(V) was present without detectable levels of α2(V). These results along with the demonstration of the presence of the α3(V) chain in some preparations of type V collagen indicated that $[\alpha 1(V)]_3$ and other molecular configurations were possible. The $[\alpha 1(V)]_3$ collagen has been identified as a product of Chinese hamster lung (CHL)[2] cells in culture (Haralson et al., 1980), and the presence of $[\alpha 1(V)]_2\alpha 2(V)$ has been extended to include several tissues (Hong et al., 1979; Welsh et al., 1980; von der Mark & von der Mark, 1979).

The location of these collagens suggests that they may function within the immediate pericellular microenvironment. Immunofluorescent staining of smooth muscle cells in culture (Miller et al., 1979) and hyaline cartilage (Gay et al., 1981) with antibodies specific for the α1(V) or B chain revealed that molecules containing this chain are deposited in close proximity to the plasma membranes. In addition, type V collagen synthesized by CHL cells in culture is found only in the cell layer where it is deposited at the periphery of the cells (Haralson et al., 1980). Others have suggested that type V collagen is a constituent of true endothelial or epithelial basement membranes (Madri & Furthmayr, 1979; Roll et al., 1980); however, this has not been borne out by chemical studies on purified basement membranes. If indeed the type V collagen does occur in basement membranes, it is not limited to these or related structures as this collagen has been isolated from such tissues as the corneal stroma where no basal lamina is present (Hong et al., 1979; Welsh et al., 1980).

In light of the continued interest in the chains of type V collagen and the need for a means of conclusively identifying these chains, we report here the isolation and characterization of the cyanogen bromide peptides of the α2(V) chain.

Materials and Methods

Preparation of α2(V) Chains. Human amniotic membranes were selected for the isolation of α2(V) chains to avoid contamination with α3(V) chains. The tissue was prepared as described elsewhere (Epstein et al., 1971; Rhodes & Miller, 1978) and digested with pepsin at 4 °C (Miller, 1972). Differential salt precipitation was used to obtain type V collagen free of other collagen types, and the α2(V) chain was isolated in pure form following chromatography under denaturing conditions on phosphocellulose (Rhodes & Miller, 1978).

CNBr Cleavage. Samples ranging from 20 to 50 mg of purified α2(V) were dissolved at a concentration of 5 mg/mL in 70% formic acid and cleaved with CNBr as described previously (Epstein et al., 1971). Following incubation for 4 h at 30 °C, the reaction mixture was diluted 10-fold with deionized water and lyophilized.

Molecular Sieve Chromatography, P-2. Following lyophilization, the CNBr cleavage products were chromatographed on a column (2.5 × 40 cm) of Bio-Gel P-2, 100–200

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[1] The nomenclature conforms to that used in two recent reviews (Bornstein & Sage, 1980; Miller & Gay, 1981).

[2] Abbreviations used: CHL, Chinese hamster lung; HPLC, high-performance liquid chromatography; CM-cellulose, carboxymethylcellulose.

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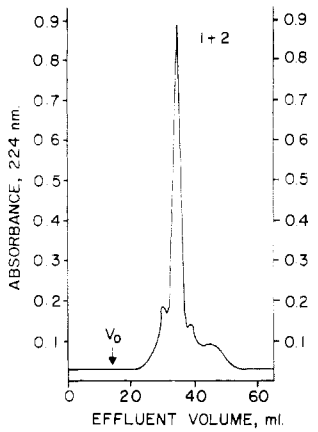

FIGURE 1: P-2 rechromatography of CNBr peptides which eluted in the included volume of the first P-2 chromatogram. The sample was dissolved in 0.5 mL of 0.01 M acetic acid and applied to a column (1.5 × 25 cm) of P-2 (200–400 mesh) equilibrated in the same solvent. Elution was achieved at 50 mL/h.

mesh, as described elsewhere (Rhodes & Miller, 1979). Appropriate fractions of the column effluent were collected and lyophilized prior to further purification. Material which eluted in the included volume of the P-2 column was rerun on a 1.5 × 25 cm column of P-2, 200–400 mesh, equilibrated in 0.01 M acetic acid at a flow rate of 50 mL/h.

CM-cellulose Chromatography. Peptides which eluted in the void volume of the P-2 column were chromatographed on a 0.9 × 9.0 cm column of CM-cellulose (CM-32, microgranular, Whatman) equilibrated in 0.005 M sodium citrate, pH 3.6 (starting buffer). Samples of up to 30 mg were dissolved in 2–5 mL of starting buffer and applied to the column maintained at 43 °C by a circulating water bath. Elution was achieved at a flow rate of 60 mL/h with a linear gradient of 0–0.16 M NaCl over a total volume of 300 mL of buffer.

Phosphocellulose Chromatography of Peptides. Phosphocellulose chromatography of peptides 3, 4, and 5 was performed as described previously (Rhodes & Miller, 1979).

Molecular Sieve Chromatography, Bio-Gel A-1.5m. Peptides recovered from CM-cellulose or phosphocellulose chromatography were rechromatographed on a calibrated column (1.5 × 140 cm) of agarose beads (Bio-Gel A-1.5m, 200–400 mesh, Bio-Rad Laboratories) equilibrated and eluted in 2.0 M guanidine hydrochloride (0.05 M Tris, pH 7.5) at a flow rate of 7.0 mL/h. Molecular weights were estimated from a standard curve constructed by using CNBr peptides from types I and II collagen (Piez, 1968).

High-Performance Liquid Chromatography. Reverse-phase high-performance liquid chromatography (HPLC)[2] was carried out by using methods previously described (Böhlen et al., 1975; Lewis et al., 1980) with some modifications. Two jacketed columns (4.5 × 260 mm each) of Lichrosorb RP-18 (Ace Scientific) in tandem were held at 44 °C and equilibrated with 0.5 M formic acid adjusted to pH 2.4 with pyridine. Peptides were dissolved in this same buffer and eluted at a flow rate of 9.5 mL/h with stepwise increases of *tert*-butyl alcohol in the starting buffer. Column eluates were monitored by using a split-stream fluorescamine detection system (Böhlen et al., 1975). Fractions of 0.75 mL were collected, and the solvent was removed by lyophilization.

Amino Acid Analysis. Purified peptides were hydrolyzed and subjected to automatic amino acid analysis as previously described (Miller, 1972). In calculations of amino acid composition, the following correction factors of given amino acids were used: 3.5% for the destruction of serine; 3%, 4.5%, and 5% for the incomplete release of valine, isoleucine, and leucine,

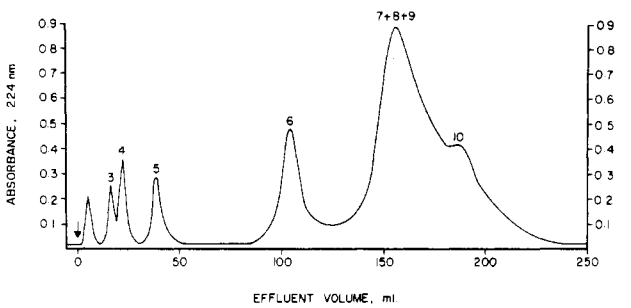

FIGURE 2: CM-cellulose elution profile of the CNBr peptides eluting in the void volume of the first P-2 chromatogram. A 30-mg aliquot of sample was dissolved in 5 mL of starting buffer (0.005 M sodium citrate, pH 3.6) and applied to a column (0.9 × 9.0 cm) of CM-cellulose maintained at 43 °C. Elution was achieved at 60 mL/h with a linear gradient of 0–0.16 M NaCl over a total volume of 300 mL of buffer. The arrow designates the initiation of the gradient.

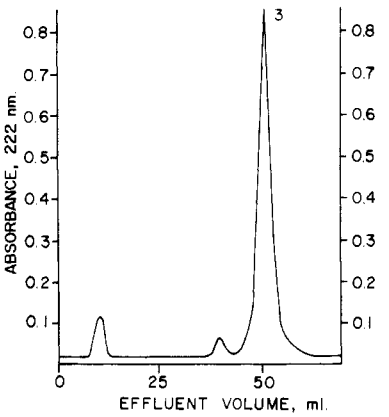

FIGURE 3: Phosphocellulose elution profile of peptide 3 recovered following CM-cellulose chromatography as depicted in Figure 2. The sample was dissolved in 0.5 mL of starting buffer (0.001 M sodium acetate, pH 3.8) and applied to a column (1.5 × 9.0) of phosphocellulose maintained at 43 °C. Elution was achieved at 50 mL/h with a linear gradient of 0–0.3 M NaCl over a total volume of 600 mL of buffer.

respectively, as determined in experiments in which the $\alpha 2$(V) chain was hydrolyzed for various intervals.

Results

Chromatography of CNBr Peptides on P-2. Initial chromatography of the CNBr peptides on P-2 revealed that the majority of the peptides eluted in the void volume of the column (figure not shown). However, a small but detectable quantity of material eluted in the included volume, and this was rechromatographed on P-2 as described under Materials and Methods. The material eluted largely as a single peak (Figure 1) which was not purified further but used for amino acid analysis. Amino acid analysis indicated the presence of two small peptides containing a total of ten residues as judged by the quantity of homoserine in the peak (Table I). The minor peaks surrounding the major peak in Figure 1 were likewise analyzed but did not contain significant quantities of peptide material.

CM-cellulose Chromatography of CNBr Peptides. Peptides which eluted in the void volume of the initial P-2 chromatogram were separated on CM-cellulose. As seen in Figure 2, the column was able to partially resolve six major fractions. Peaks containing peptides 3, 4, and 5 were each pooled separately, desalted, lyophilized, and rechromatographed on phosphocellulose (see below). The peaks containing peptides 6, 7 + 8 + 9, and 10 were also pooled, desalted, and rechromatographed on agarose.

Table I: Amino Acid Composition[a] and Molecular Weight of the α2(V) Collagen Chain CNBr Peptides (1–10)

amino acid	1, 2	3	4	5	6	7	8	9	10	total CNBr peptides	α2(V) chain[b]
3-Hyp[c]	0	0	t[f]	t	0	0.8	t	2.0	0	3	4
4-Hyp[c]	0	3.0	3.8	2.6	9.2	23.0	25.2	27.0	13.8	108	110
Asp	0	1 (1.0)	1 (1.0)	2 (2.0)	3 (3.0)	12	8 (8.0)	16	5 (5.1)	48	46
Thr	0	0	1 (1.0)	2 (2.1)	1 (1.0)	7 (7.1)	8 (7.9)	7 (7.0)	2 (1.9)	28	28
Ser	1 (1.0)	2 (1.8)	1 (1.0)	2 (1.8)	3 (3.0)	6 (6.0)	10	7 (7.0)	3 (2.8)	35	33
Glu	0	0	4 (4.2)	1 (1.1)	7 (7.1)	16	20	16	11	75	78
Pro[c]	0	1.3	4.9	4.1	7 (7.0)	22.1	22.0	30.0	9.3	101	98
Gly	3 (3.0)	6 (5.8)	11	11	28	72	72	83	36	322	329
Ala	1 (1.0)	0	1 (1.0)	1 (1.0)	5 (5.2)	14	10	14	5 (4.8)	51	49
Val	2 (2.0)	0	2 (2.0)	0	2 (2.1)	6 (5.9)	6 (5.9)	8 (8.0)	4 (4.0)	30	30
Ile	0	1 (1.0)	0	0	1 (1.1)	2 (2.2)	5 (4.9)	4 (3.8)	1 (1.0)	14	13
Leu	1 (1.0)	1 (1.0)	1 (1.0)	1 (1.0)	3 (3.0)	7 (6.8)	6 (6.0)	10	4 (4.3)	34	32
Tyr	0	0	0	0	0	0	0	0	0	0	0
Phe	0	1 (1.0)	0	0	2 (2.0)	2 (2.0)	2 (2.2)	3 (2.9)	1 (1.0)	11	10
Hyl[c]	0	0	0	0.9	3.6	5.5	5 (5.1)	2.8	2.0	20	21
Lys[c]	0	0	0	0.1	1.2	1.4	2.7	3.7	2.6	12	9
His	0	0	0	1 (1.0)	2 (1.9)	2 (2.0)	1.2	3 (3.1)	0	9	12
Arg	0	0	1 (1.0)	0	5 (5.0)	11	11	14	4 (4.2)	46	46
Hse	2 (2.0)	1 (0.9)	1 (0.9)	1 (0.9)	1 (0.9)	1 (0.8)	1 (0.8)	0	1 (0.8)	9	10[d]
total	10	17	33	30	84	211	215	251	105	956	958
M_r by amino acid analysis	990	1573	3060	2688	7869	19830	20195	23515	9866	89686	
M_r by agarose chromatography	990[e]	2000	3300	3100	8000	20500	20500	20500	11000	89190	

[a] Residues per peptide to the nearest whole number. Actual values given in parentheses where less than ten residues are present. [b] Calculated from amino acid analyses for a chain comprised of 956 residues. [c] Actual values listed due to incomplete hydroxylation of prolyl and lysyl residues. [d] Represents methionine in the intact chain. [e] Molecular weight determined by amino acid analysis only. [f] t represents trace amounts present which are too small to accurately quantitate.

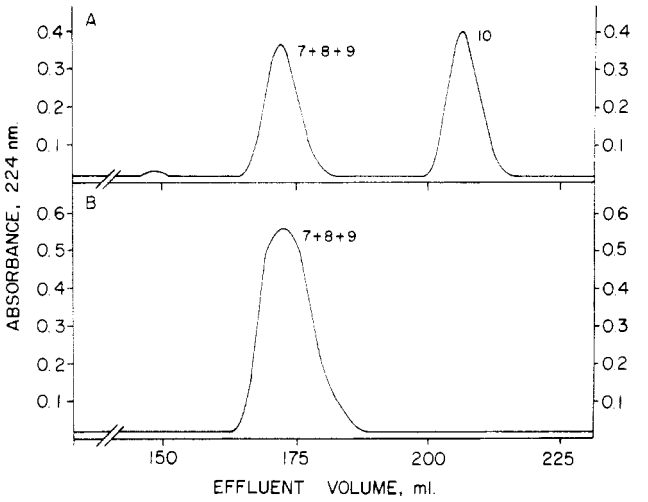

FIGURE 4: Bio-Gel A-1.5m elution profile of certain peptides recovered after CM-cellulose chromatography. The column was equilibrated and eluted with 2.0 M guanidine hydrochloride (0.05 M Tris, pH 7.5) at 6.8 mL/h. (A) The elution profile of peptide 10; (B) the elution profile of the peak containing peptides 7, 8, and 9.

Phosphocellulose Chromatography. Peptide 3 when rechromatographed on phosphocellulose gave a single homogeneous peak as seen in Figure 3. Similar patterns were obtained upon rechromatography of peptides 4 and 5.

Molecular Sieve Chromatography, Bio-Gel A-1.5m. Peptides 6, 7 + 8 + 9, and 10 recovered from CM-cellulose and 3, 4, and 5 retrieved from phosphocellulose were each rechromatographed on a column of Bio-Gel A-1.5m. Peptides 3, 4, 5, and 6 each gave single peaks exhibiting an M_r of 2K, 3.3K, 3.1K, and 11K, respectively (figures not shown). The agarose elution profile of peptide 10 revealed the presence of overlapping material derived from the peak containing peptides 7 + 8 + 9. However, peptide 10 was well resolved from the higher molecular weight peptides and eluted at a position corresponding to an M_r of 8000 (Figure 4A). Following purification on agarose, peptides 3, 4, 5, 6, and 10 were subjected to amino acid analysis.

The peak containing peptides 7 + 8 + 9 accounted for 60% of the total material recovered from CM-cellulose and slightly more than 50% of the total amount of material among the CNBr cleavage products of α2(V). Thus, the elution of essentially all of this material as a single peak indicating an M_r of 20 500 from agarose was unexpected (Figure 4B). However, the agarose peak was noticeably broadened when compared to the elution profile of other peptides. These observations as well as the shape of the CM-cellulose peak and the proportion of material recovered in the peak suggested that it contained a mixture of peptides of similar molecular weight and charge. Additional attempts to resolve the peptides with a variety of gradient conditions by using CM-cellulose and phosphocellulose proved unsuccessful. Resolution and purification of the peptides were thus achieved by using HPLC.

HPLC of Peptides 7, 8, and 9. Separation of peptides 7, 8, and 9 was initially attempted by using 1-propanol gradients in formic acid–pyridine buffer. No resolution was achieved by operating in the pH range of 4.0–5.5. Lowering the pH to 3.0 resulted in partial resolution of two peaks with an RP-8 column, and this separation was improved by using an RP-18 column eluted at pH 2.4. Use of stepwise elution with *tert*-butyl alcohol in place of the 1-propanol, as described under Materials and Methods, achieved essentially complete separation of three peptides as seen in Figure 5. Each of these peptides was recovered and analyzed for amino acid composition. The shoulders on the front of peptides 7 and 9 gave

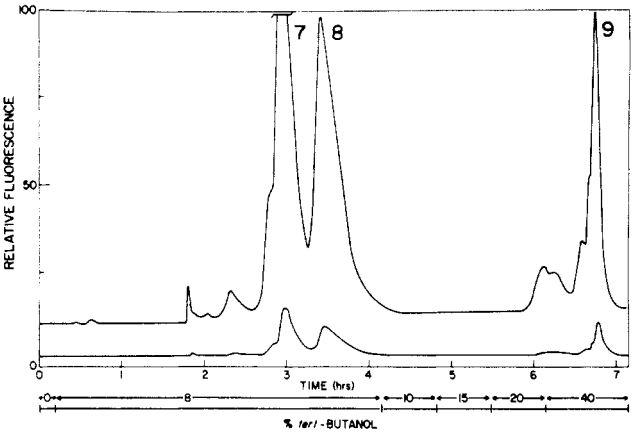

FIGURE 5: HPLC fractionation of CNBr peptides 7, 8, and 9. The sample (2.4 mg) was dissolved in 0.5 mL of formic acid adjusted to pH 2.4 with pyridine and applied to two tandemly arranged RP-18 columns maintained at 44 °C. Stepwise elution of the columns with *tert*-butyl alcohol was performed at a flow rate of 9.5 mL/h. Fractions of 0.75 mL were collected, and approximately 0.5% of the effluent was diverted to a fluorescamine detector system. Fluorescence was recorded at high sensitivity (top curve) and a sensitivity 10 times lower (botton curve).

analyses virtually identical with the main peak, indicating some chromatographic heterogeneity for the peptides when chromatographed in this system.

Amino Acid Composition. The amino acid composition of the peptides recovered from CNBr digestion of the α2(V) chain is presented in Table I. The data indicate that the peptides have been isolated in essentially pure form. The peptides account for a chain of about 956 amino acid residues, ranging in M_r from less than 1000 to over 23 000. The total number of amino acids recovered in the peptides is slightly less than that commonly observed for a collagen α chain and may reflect the loss of a small portion of the α2(V) chain sequence when molecules containing this chain were exposed to limited digestion with pepsin. The data further establish the uniqueness of each peptide and thus the unique nature of the α2(V) chain. Each of the major peptides also appears to be from a potentially helical domain as evidenced by one-third glycine content. In addition, each peptide was recovered in approximately equimolar proportions as judged by recovery following chromatography and quantitation of homoserine or other appropriate amino acids (data not shown).

Discussion

The data obtained in this study have shown that the α2(V) chain, recovered from collagen brought into solution by means of limited pepsin digestion, yields ten unique CNBr peptides which account within experimental error for all the amino acids of the entire chain. The total number of amino acids recovered in the CNBr peptides is 956 residues, suggesting either a decrease in size for the helical domain in this chain or a pepsin-susceptible region within the helical domain. In any event, these results are consistent with the observation that a related chain, α1(V), as recovered following limited pepsin digestion contains approximately 1018 amino acid residues (Rhodes & Miller, 1979) and that the α2(V) chain exhibits a somewhat smaller apparent M_r than α1(V) when evaluated by disc gel electrophoresis (Burgeson et al., 1976; Rhodes & Miller, 1978) as well as by molecular sieve chromatography (Rhodes & Miller, 1978).

Characterization of the CNBr peptides of other collagen chains has led to the observation that homology exists among several peptides in terms of size, sequence, and position within the chain (Fietzek & Kühn, 1976; Bornstein & Traub, 1979).

In this regard, it is interesting to note that several of the α2(V) chain peptides are similar in size and, in one instance, similar in amino acid composition to CNBr peptides derived from other collagen chains. Peptide 3 is the same size as peptide 2 from the α2(IV) chain (Dixit & Kang, 1980). Peptide 4 contains the same number of residues as α1(II)-CB6 (Miller & Lunde, 1973) and is also similar in size to α2(IV)-CB7 (Dixit & Kang, (1980). Two previously described peptides, α2(I)-CB2 (Click & Bornstein, 1970; Epstein et al., 1971) and α2(IV)-CB7 (Dixit & Kang, 1980) could be homologous to peptide 5 of the α2(V) chain on the basis of size. Peptide 6, like α1(V)-CB4 (Rhodes & Miller, 1979) and α1(II)-CB12 (Miller & Lunde, 1973), contains 84 residues and also bears some compositional resemblance to α1(II)-CB12. Peptides 7 and 8 are approximately the same length, and thus comparisons for a possible homology based on size hold true for both peptides. They are similar in size to α1(I)-CB6 (Click & Bornstein, 1970; Epstein et al., 1971), α2(IV)-CB4 (Dixit & Kang, 1980), α1(V)-CB3, and α1(V)-CB6 (Rhodes & Miller, 1979). However, it is apparent that no homology would exist with peptides α1(I)-CB6 and α2(IV)-CB4 as these are the carboxy-terminal peptides of their respective chains. Peptide 9, the carboxy-terminal peptide of the α2(V) chain, shows no obvious homology with any of the C-terminal peptides of other collagen chains. Confirmation of true homologies which may exist will require more information with regard to the positioning of the peptides within the chain and the primary sequence of the peptides. It will be of interest to note as this information becomes available whether homologies which may exist are more prevalent between the type V collagen chains as compared to homologies between the type V chains and the interstitial and basement membrane collagen chains.

Of particular interest in the characterization of the α2(V) chain has been the usefulness of HPLC in the purification and characterization of the CNBr peptides. Unlike the peptides of most collagen chains, the three peptides 7, 8, and 9 could not be purified by using conventional ion-exchange and molecular sieve procedures. The greater resolving capacity of HPLC resulted in the separation of three peptides of essentially the same size with only very slight differences in their charge properties. Thus, the technique can readily be used, not only for peptide fingerprints of collagen (van der Rest et al., 1977; Black et al., 1980) but also, as demonstrated here, as a valuable preparative procedure for peptides which are difficult to purify to homogeneity. In addition, the recoveries from HPLC are generally high (Lewis et al., 1980), and with the high sensitivity of the fluorescamine detection system (Böhlen et al., 1975) very little sample is lost in monitoring elution. These advantages will make HPLC a valuable tool for the purification and characterization of collagen chains and peptides which may constitute a relatively small proportion of the total tissue collagen.

Acknowledgments

We thank S. C. Sun, Margaret S. Vail, and Chris Zahm for their expert technical assistance and Julia Sanders for typing the manuscript and preparing the figures.

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