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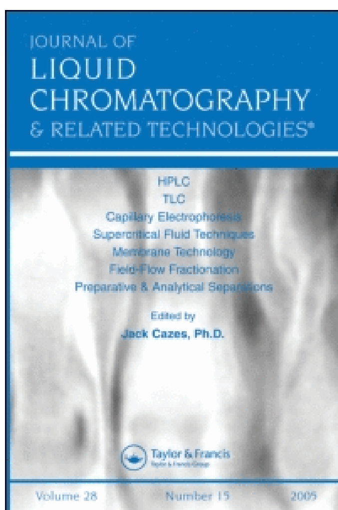

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SEPARATION OF HMG PROTEINS BY REVERSE-PHASE HPLC

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ABSTRACT

A high-pressure liquid chromatographic method is described for separating the high mobility group (HMG) proteins on a 5-μm Nucleosil C18 column with the use of the ion pairing agent trifluoroacetic acid (TFA) in the mobile phase. With a multistep acetonitrile-TFA gradient, the calf thymus HMG proteins elute from this column as separate peaks in the order HMG 17, 14, 2, and 1. Protein elution is monitored by measuring the absorbance at 214 nm in a 3-mm flow cell, and the identity and purity of the peaks are confirmed by polyacrylamide gel electrophoresis and amino acid analysis. Both HMG 1 and 2 elute as multiple peaks, suggesting the presence of major variants of these two proteins. Other peaks in the chromatogram include degradation products of HMG 1 and histone H1.

INTRODUCTION

One subset of nonhistone proteins that has been isolated and studied in considerable detail during the past several years is the high mobility group, or HMG, proteins. Unlike the histones, HMG proteins are deficient in basic amino acids

and rich in acidic amino acids and proline. They are extracted from chromatin in 0.35 M sodium chloride and remain soluble in 2% trichloroacetic acid (TCA).

Four proteins, HMG 1, 2, 14, and 17, have been identified by gel electrophoresis and column chromatography as the primary HMG components associated with chromatin in a variety of somatic tissues. Numerous recent reports (1-4) have demonstrated that two of these proteins, HMG 14 and 17, may be preferentially associated with active chromatin, and that they may somehow modulate changes in chromatin structure required for gene activation. Various other proteins and peptides that coextract with the HMGs, e.g., ubiquitin and proteolytic fragments of HMG 1 and H1 histone, have also been identified (5,6).

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Existing methods for separating these proteins preparatively require a combination of ion exchange chromatography and selective TCA precipitation (6). While this approach is reliable, reproducible, and permits the isolation of large quantities of at least two of the HMG proteins (HMG 1 and 2), sample preparation and chromatography times are extensive. To separate all four HMGs, additional fractionation steps (such as selective TCA precipitation) must be performed on fractions that coelute with HMG or histone degradation products.

To circumvent these difficulties, we have devised a rapid (80-min) preparative method for separating the HMG proteins by reverse-phase high-pressure liquid chromatography (HPLC) on Nucleosil 100, a spherical octadecyl-silicic acid support, using the ion pairing agent trifluoroacetic acid (TFA) in the mobile phase. With this approach, each of the major HMG proteins and specific proteolytic fragments of H1 histone and HMG 1 are resolved into separate, well defined peaks. In addition, both HMG 1 and 2 resolve into at least two distinct

peaks. Amino acid analyses suggest that the HMG 1 species may represent sequence variants that differ in the number of their glutamic acid and alanine residues, while the two HMG 2 proteins are probably not sequence variants.

MATERIALS AND METHODS

HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ). Lots were chosen that had a relative absorbance (vs water) at 200 nm (in a 1-cm cell) of 0.017 or lower. Trifluoroacetic acid was obtained from Eastman Kodak Co. (Rochester, NY).

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Total HMG protein was extracted from calf thymus using the method of Bhullar and Candido (7). Approximately 10 mg was dissolved in Buffer A (aqueous 0.1% TFA), and centrifuged prior to injection to remove insoluble material. The proteins were separated on a 5-μm Nucleosil 100 column using a multistep acetonitrile gradient. The mobile phase was initiated at 20% Buffer B (40% acetonitrile, 0.1% TFA), and the Buffer B concentration was increased at a rate of 1.33%/min for 30 min to a final 60% Buffer B. After 30 min, the rate of Buffer B addition was then changed to 15%/min for 1 min, 0.43%/min for 35 min, and finally 10%/min for 1 min. Eluent emerging from the column was routed to the 214-nm detector, fractions collected, and the pooled samples lyophilized.

The separations were performed on an Altex Model 332 liquid chromatograph (Altex Scientific, Berkeley, CA), using two Model 110A pumps, a microprocessor-controlled solvent programmer (Model 410), and a Pharmacia UV-1/214 detector fitted with a 3-mm flow cell (Piscataway, NJ). Ten grams of Nucleosil 100 (5 μm) in methanol was slurry-packed into a 7.8 x 300 mm column fitted with 2-μm frits, by using a pneumatic pump (Jones Chromatography Co., Stoe, OH) at 6000 psi for 30 min. A

precolumn (4.5 x 250 mm, dry-packed with 37-53 μm precolumn silica, Whatman Chemical Co., Clifton, NJ) was installed before the injector, and a guard column (4.5 x 70 mm, dry-packed with 30 μm C18-derivatized silica particles, Serva Biochemical Co., Long Island, NY) was placed before the Nucleosil column.

After separation, the isolated HMG fractions were identified by electrophoresis (3 h, 130 V, 20 mA) in 10-cm acid-urea gels (8) containing 2.5 M urea. Amino acid analyses of each fraction were also obtained on a Beckman 120C amino acid analyzer following hydrolysis of the lyophilized proteins in 4 M methanesulfonic acid at 110 °C for 40 h.

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RESULTS AND DISCUSSION

Eight major and several minor peaks were obtained following chromatography of total HMG protein on Nucleosil 100, using the multistep acetonitrile gradient shown in Fig. 1. The proteins in each peak were identified by collecting fractions across the middle of the peak (to limit contamination from adjacent peaks) and subjecting the isolated (lyophilized) proteins to electrophoresis in acid-urea gels and amino acid analysis.

HMG 17, identified by its electrophoretic mobility in gels and by its characteristic amino acid composition (Table 1), eluted as the first major peak (peak A) at approximately 55% Buffer B. HMG 14, a minor component in the HMG preparation used in these experiments, eluted as a small peak (peak B) 5 min later. The amino acid compositions of these two HMG peaks were essentially identical to those reported previously for HMG 14 and 17 (9).

Peak C, which eluted at 40 min, did not contain an identifiable HMG protein but contained numerous peptides that migrated throughout the length of the gel. This peak was substantially larger in older (6-month) HMG samples that showed

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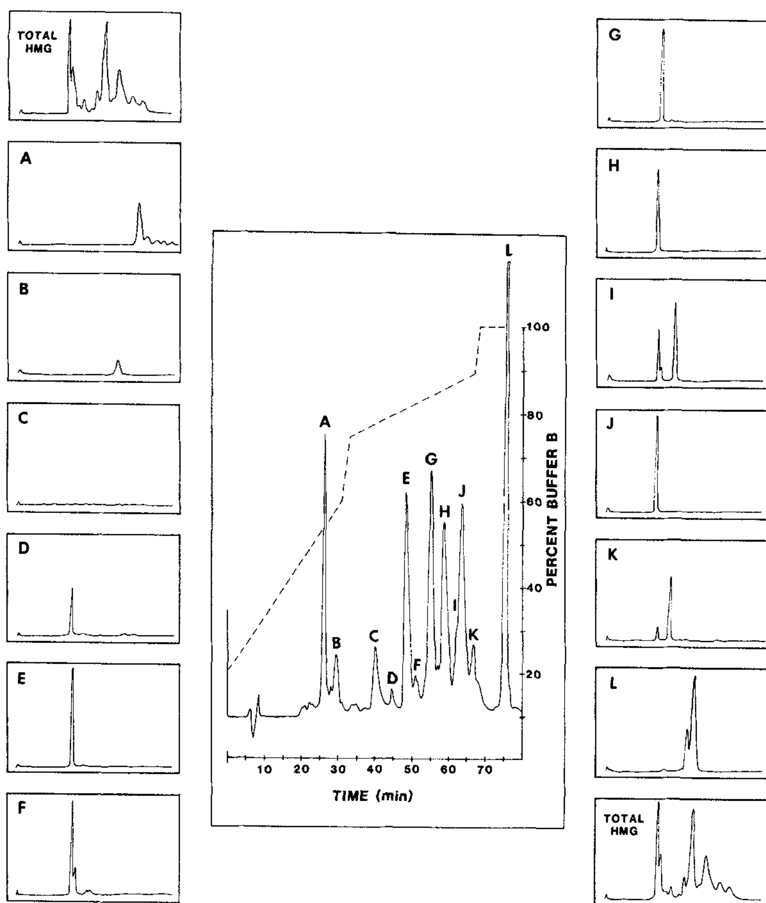

Figure 1 Separation of HMG proteins by HPLC and analysis of the isolated fractions by disc gel electrophoresis. CENTER PANEL: Absorption profile (214 nm) of total HMG protein separation on a Nucleosil 100 column as described in Materials and Methods. Dashed line: Buffer B gradient 100% Buffer B is 40% acetonitrile; 0.1% TFA. PERIPHERAL PANELS: Microdensitometer scans of Naphthol blue black stained HMG fractions following electrophoresis in acid-urea gels at 120 V for 3 hr. The labeled fractions in these panels correspond to the isolated HPLC peaks in the center panel. Electrophoresis is from the left (cathode) to right (anode). Peak A: HMG 17; Peak B: HMG 14; Peak C: numerous peptides of various sizes; Peak D: HMG 1; Peaks E and G: HMG 2; Peaks F and I: HMGs 1, 2, and 3; Peaks H and J: HMG 1; Peak K: HMG 3, the N-terminal end of HMG 1; and Peak L: fragments of H1 histone.

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TABLE 1
Amino Acid Compositions† of Peaks Obtained Following HPLC of Total HMG Protein on Nucleosil 100

Peak*:	A	Expected	B	Expected	E	G	Expected	H	J	Expected	K	L
HMG:	17	17	14	14	2	2	2	1	1	1	3	8
Lysine	24.5	24.3	21.9	21.1	19.9	19.5	19.4	20.9	21.1	21.3	18.4	20.4
Histidine	0	0	0	0.2	1.7	1.8	2.0	1.3	1.2	1.7	1.4	0.1
Arginine	4.4	4.1	5.3	5.4	4.2	4.2	4.7	3.6	3.5	3.9	3.7	3.1
Aspartic acid	10.9	12.0	8.0	8.3	9.9	10.7	9.3	10.9	10.3	10.7	9.3	3.6
Threonine	1.7	1.2	4.1	4.1	2.1	2.3	2.7	2.3	2.6	2.5	4.0	5.2
Serine	2.9	2.3	7.3	8.0	7.6	7.9	7.4	5.0	5.0	5.0	5.2	8.3
Glutamic acid	11.6	10.5	18.1	17.5	17.6	17.3	17.5	18.4	17.0	18.1	11.6	5.8
Proline	12.7	12.9	9.0	8.1	7.9	7.8	8.9	6.7	6.8	7.0	7.5	7.2
Glycine	9.7	11.2	6.5	6.4	6.6	6.5	6.5	5.4	5.4	5.3	6.7	8.5
Alanine	18.5	18.4	14.3	14.8	7.6	7.5	8.1	9.0	10.8	9.0	10.6	21.2
Half-cystine	0	0	0	0	0.3	0	trace	0.6	0	trace	0	0
Valine	2.0	2.0	3.9	4.0	1.7	1.7	2.3	2.0	2.1	1.9	6.6	6.1
Methionine	0	0	0	0.1	1.8	1.8	0.4	1.9	1.9	1.5	1.8	0
Isoleucine	0	0	0	0.3	1.2	1.2	1.3	1.7	1.7	1.8	2.4	1.4
Leucine	1.0	1.0	1.8	2.0	2.1	2.4	2.0	2.1	2.3	2.2	4.2	7.4
Tyrosine	0	0	0	0.2	2.9	2.9	2.0	3.2	3.1	2.9	2.8	0.9
Phenylalanine	0	0	0	0.3	4.1	3.7	3.0	4.2	4.1	3.6	3.7	0.8
Tryptophan	0	0	0	nd	0.7	0.8	nd	0.8	0.9	nd	0	0

*Letter description as shown in Figure 1.
† Amino acid analyses of each fraction were obtained on a Beckman 120C amino acid analyzer following hydrolysis of the lyophilized proteins in 4 M methanesulfonic acid at 110°C for 40 h. Expected compositions are those published by Johns (9).

signs of degradation by gel electrophoresis, suggesting that these peptides are fragments of histone or HMG proteins.

Two well separated peaks eluting between 80 and 85% Buffer B at 48 min (peak E), and 55 min (peak G), respectively, were found to contain a protein with an electrophoretic mobility and amino acid composition characteristic of HMG 2. Except for minor differences in their aspartic acid and half-cystine contents, the amino acid compositions of the proteins in these two fractions were virtually identical. Thus these proteins may not be amino acid sequence variants of HMG 2. They may, however, differ in their extent of amidination (number of glutamine or asparagine residues) or degree of modification by acetylation, phosphorylation, or methylation. Multiple species of calf thymus HMG 2 were also observed by Goodwin _et al_. (6,10,11) following chromatography of whole HMG 2 on carboxymethyl-sephadex. Significant differences in the amino acid compositions of the two major and two minor HMG 2 species were not detected in their analyses either.

Similarly, three peaks were found to contain proteins that coelectrophoresed with HMG 1. The amino acid compositions (Table 1) of the proteins in the two larger peaks, H and J, were also characteristic of HMG 1 (sufficient sample was not available for analyzing peak D). The proteins in these two peaks differed only in their glutamic acid, alanine, and half-cystine contents. Although these differences were small, they suggest the possibility that the two major HMG 1 species observed here may be amino acid sequence variants. The presence of several HMG 1 species was suggested earlier by Goodwin _et al_. (11), and was based on their observation that the isoelectric focusing pattern of total HMG 1 contained multiple peaks. It was thought that the complexity might arise as a result of aggregation. Multiple species were never obtained by chromatography.

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Minor peaks F and I were found to contain a mixture of HMG 1, 2, and 3. HMG 3, the N-terminal end of HMG 1, eluted as the major component of peak K at 67 min. Peak I, which contained considerable HMG 3 (probably because of incomplete separation of adjacent peaks upon collection), also contained a small amount of HMG 2. This protein (HMG 2) could not be accounted for by overlap from adjacent peaks (since peaks H and J are both HMG 1), and its presence suggests that another HMG 2 variant may exist and elute as a shoulder (labeled I in Fig. 1) on peak J. Similar results were observed for peak F. The major component of this peak was HMG 1, which suggests that this peak is a minor HMG 1 variant that elutes beween two HMG 2 variants.

The last peak, L, eluted from the column at 75 min and 100% Buffer B. The proteins (at least two) in this fraction displayed an electrophoretic mobility midway between HMG 2 and HMG 17, similar to that observed for fragments of H1 histone. The acetonitrile concentration at which this peak elutes (40%) is very close to that observed for intact H1 histone (12). Amino acid analysis of this peak (Table I) also supports the idea that these proteins are fragments of H1; the protein is rich in lysine and alanine and low in glutamic and aspartic acids.

This approach offers several advantages over currently used methods for separating and analyzing the HMG proteins. The method is rapid, requiring only 80 min per run. Each HMG protein, several potential HMG variants, and various contaminating histone H1 and HMG degradation products all separate as distinct peaks in one chromatographic run. Using the preparative column, several milligrams of each HMG protein may be obtained without sacrificing the quality of the resolution. Separations may be performed in the analytical mode by using an analytical or microbore column and a 1-cm flow

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cell, or by postcolumn derivatization of the proteins with a fluorescent tag. Because intact histones begin eluting at an acetonitrile concentration greater than that required for elution of the final protein in the HMG fraction (peak L, H1 peptides), this approach may be modified to permit the separation of both histones (12) and HMG proteins in a single run. By using entirely volatile buffers, extensive and time-consuming dialysis steps are eliminated, and the isolated fractions may be lyophilized directly.

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REFERENCES

1. Weisbrod, S. and Weintraub, H., Isolation of a Subclass of Nuclear Proteins Responsible for Conferring a DNase I-Sensitive Structure on Globin Chromatin, Proc. Natl. Acad. Sci. USA 76, 630, 1979.
2. Sandeen, G., Wood, W.I., and Felsenfeld, G., The Interaction of High Mobility Proteins HMG 14 and 17 with Nucleosomes, Nucleic Acids Res. 8, 3757, 1980.
3. Reeves, R. and Candido, E.P.M., Partial Inhibition of Histone Deacetylase in Active Chromatin by HMG 14 and HMG 17, Nucleic Acids Res. 8, 1947, 1980.
4. Weisbrod, S., Grandine, M., and Weintraub, H., Interaction of HMG 14 and 17 with Actively Transcribed Genes, Cell 19, 289, 1980.
5. Walker, J.M., Goodwin, G.H., and Johns, E.W., The Isolation and Identificatio of Ubiquitin from the High Mobility Group (HMG) Non-Histone Protein Fraction, Febs Letters 90, 327, 1978.
6. Goodwin, G.H. and Johns, E.W., The Isolation and Purification of the High Mobility Group (HMG) Nonhistone Chromosomal Proteins, in Methods in Cell Biology, Stein, G., Stein, J., and Kleinsmith, L.J., eds, Vol. 16, Academic Press, New York, 1978, pp. 257.
7. Bhullar, B.S. and Candido, E.P.M., An Alternative Procedure for the Isolation of High Mobility Group Proteins, Anal. Biochem. 118, 247-251, 1981.

8. Panyim, S., Bilek, D., and Chalkley, R. An Electrophoretic Comparison of Vertebrate Histones, J. Biol. Chem. 246, 4206, (1971).
9. Johns, E.W., HMG Chromosomal Proteins, Academic Press, New York, 1982.
10. Mathew, C.G.P., Goodwin, G.H., Gooderham, K., Walker, J.M., and Johns, E.W., A Comparison of the High Mobility Group Non-Histone Chromatin Protein HMG2 in Chicken Thymus and Erythrocytes, Biochem. Biophys. Res. Commun. 87, 1243, 1979.
11. Goodwin, G.H., Nicholas, R.H., and Johns, E.W., Microketerogeneity in a Non-Histone Chromosomal Protein, Febs Letters 64, 412, 1976.
12. Mazrimas, J.A. and Balhorn, R., Reversed-Phase HPLC Separation of Somatic Histones, Liquid Chromatography Magazine, 1, 104, 1983.

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