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METHOD FOR COMPLETE SEPARATION OF THE HIGH MOBILITY GROUP (HMG) PROTEINS HMG I AND HMG Y FROM HMG 14 AND HMG 17 AND A PROCEDURE FOR PURIFICATION OF HMG I AND HMG Y

TERJE LUND, JOSTEIN HOLTLUND, BJØRN SKÅLHEGG and SØREN G. LALAND*
Department of Biochemistry, University of Oslo, P.O. Box 1041, Blindern, 0316 Oslo 3 (Norway)
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SUMMARY

A purification procedure which separates the four low-molecular-weight high mobility group (HMG) proteins, HMG 14, 17, I and Y, is described. The procedure includes chromatography on phosphocellulose and Blue Sepharose combined with reversed-phase high-performance liquid chromatography. The blue Sepharose column separates HMG I and Y completely from HMG 14 and 17, and should therefore be an useful tool for the identification of these proteins which in several reports have been confused with HMG 14 and 17. HMG I and Y on the one hand and HMG 14 and 17 on the other exhibited considerable differences in their affinities for Blue Sepharose, probably reflecting fundamental differences in biological function.

INTRODUCTION

The high mobility group (HMG) non-histone chromosomal proteins are a group of relatively abundant chromatin-associated proteins present in most eucaryotic cells[1]. They are characterized by their solubility in 5% perchloric acid (PCA) and 2% trichloroacetic acid (TCA) and their high content of basic and acidic amino acid residues, as well as an high content of proline. This unusual amino acid composition is the main reason for the lack of secondary structure in parts of the molecules[1].

The HMG proteins can be divided into two subgroups: the high-molecular-weight proteins consisting of HMG 1 and 2 with molecular weight (MW) of approximately 28 000, and the low-molecular-weight group consisting of HMG 14 and 17 with MW of about 10 000[2]. In addition to these well characterized HMG proteins, two novel HMG proteins, of the low-molecular-weight type have recently been reported by this laboratory[3–5]. These two proteins, designated HMG I and Y, seem to be characteristic of, or at least are expressed at elevated levels, in proliferating cells[3–6]. They are both phosphorylated in interphase and superphosphorylated in metaphase and exhibit a high affinity for A-T rich DNA sequences[7,8]. The two novel small HMG proteins are of interest due to their apparent correlation with cell proliferation[6]

When analysing the group of small HMG proteins, one has to consider four polypeptide chains with very similar molecular weights and amino acid compositions, resulting in similar migration velocities on both sodium dodecyl sulphate (SDS) and acetic acid–urea gels and a concomitant risk of misinterpretation of the gel patterns. For instance, in several reports on phosphorylation of HMG proteins *in vivo*, HMG I and Y have been mistaken for phosphorylated HMG 14 and 17[9–11] We therefore considered it important to develop a simple method for separating the closely related HMG I and Y from HMG 14 and 17. This paper describes how this separation can be achieved by chromatography on Blue Sepharose, and in addition the purification to apparent homogeneity of all four small HMG proteins by including chromatography on phosphocellulose and reversed-phase high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Propagation of cells

HeLa S3-cells were propagated in suspension culture containing Eagles minimum essential medium with 10% foetal calf serum, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) buffer (pH 7.3) and non-essential amino acids.

Extraction of HMG proteins

The cells (usually 10^9) were extracted twice with 5% PCA, and the acid-soluble proteins were precipitated with TCA (final concn. 20%). TCA-precipitated proteins were washed with acetone–conc. hydrochloric acid (400:1), followed by acetone–0.1 *M* hydrochloric acid (6:1) and finally three times with acetone. The protein pellet was suspended in 5% PCA, 1 volume of acetone was added and the precipitated material was removed by centrifugation. To the supernatant were added 6 volumes of acetone–0.07 *M* hydrochloric acid, and the resulting precipitate was washed with acetone–0.1 *M* hydrochloric acid (6:1) and three times with acetone. This protein fraction, which contains the PCA-soluble HMG proteins, was used for further purifications.

Purification by phosphocellulose chromatography

PCA-soluble proteins were dissolved in 10 ml of 0.4 *M* sodium chloride, 10 m*M* Tris–HCl (pH 8.0) and applied to a Whatman P 11 phosphocellulose column (7 cm × 1.9 cm) equilibrated with the same buffer. Chromatography was carried out with a linear sodium chloride gradient as indicated in the legends, at a flow-rate of 1.3 ml/min. The eluate was monitored with a zinc lamp (Pharmacia) having a 214-nm filter and collected in fractions of 4 ml.

Purification by Blue Sepharose chromatography

The selected fractions from the phosphocellulose column were diluted to 0.2 *M* sodium chloride in 10 m*M* Tris–HCl (pH 8.0) and aplied to a Blue Sepharose CL-6B (Pharmacia) column (5 cm × 1.5 cm), equilibrated with the same buffer. The proteins were eluted with a sodium chloride gradient as indicated in the legends, at a flow-rate of 1.3 ml/min. The eluate was monitored at 214 nm, collected in fractions of 4 ml and the proteins were subsequently precipitated with TCA (final concn. 20%).

Purification by reversed-phase HPLC

Crude PCA-soluble proteins or partially purified HMG proteins were dissolved in 0.01% trifluoroacetic acid (TFA), and applied to an Ultrapore RPSC *n*-propyldimethyl silane column (Beckman) (75 mm × 4.6 mm for analytical use and 250 mm × 10 mm for preparative purposes), which was equilibrated with 0.01% TFA. The elution was a modification of that described by Anzano *et al.*[12], using a linear acetonitrile gradient in 0.01% TFA, with 0% acetonitrile at time 0 and 35% at 50 min, at flow-rates of 1 ml/min for the small column and 4 ml/min for the large column. The eluate was monitored with a Kontron Uvikon 725 spectrophotometer at 220 nm, the eluted protein was collected manually, and pooled fractions were dried under vacuum.

Polyacrylamide gel electrophoresis (PAGE)

Proteins were analysed by electrophoresis in polyacrylamide slab gels (13 cm × 13 cm × 0.1 cm), with a resolving gel (15% acrylamide) as described by Panyim and Chalkley[13], and a stacking gel as described by Spiker[14].

RESULTS

The aim of the present work was to establish a simple and safe procedure for the separation and identification of four related HMG proteins, HMG 14, 17, I and Y. The purified proteins were analysed by acetic acid–urea PAGE, which gives a better separation of these proteins than SDS-PAGE.

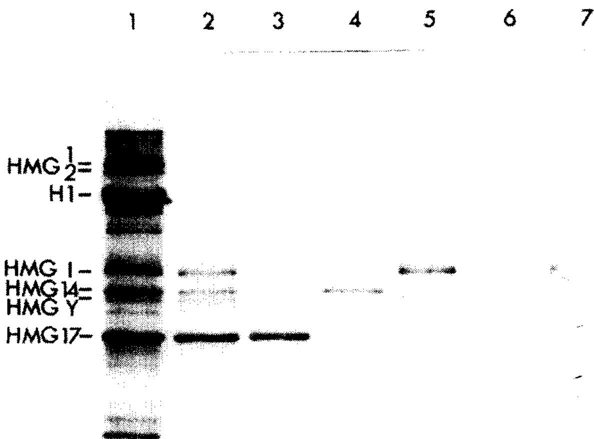

Fig. 1. Acetic acid–urea PAGE of HMG proteins at different levels of purification. Lanes: 1 = total PCA-extracted proteins from HeLa S3-cells; 2 = fractions 23–35 from phosphocellulose chromatography; 3 = fractions 14–17 from Blue Sepharose chromatography; 4 = fractions 21–24 from Blue Sepharose chromatography; 5 = fractions 34–36 from Blue Sepharose chromatography; 6 = HMG Y purified by HPLC, 7 = HMG I purified by HPLC.

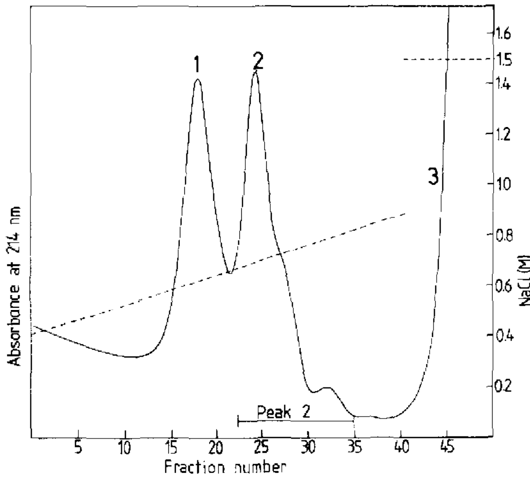

Fig. 2. Chromatography on phosphocellulose of PCA-soluble proteins from HeLa S3-cells. The chromatography was carried out with a sodium chloride gradient. ———, Absorbance at 214 nm; ---------, sodium chloride concentration.

HeLa S3-cells, which contain relatively high levels of low-molecular-weight HMG proteins, were used as the source for the purifications. In the PCA extract from HeLa S3-cells the amounts of HMG I and 14 are approximately one third that of HMG 17, and the amount of HMG Y is about one ninth that of HMG 17, judged from scans of Coomassie Brilliant Blue-stained acetic acid–urea gels (Fig. 1, lane 1, scans not shown).

The low-molecular-weight HMG proteins were separated from most of the other PCA-soluble proteins, including histone H1, by chromatography on phosphocellulose with a linear gradient ranging from 0.4 to 0.9 *M* sodium chloride (Fig. 2). The proteins which remained bound were step-eluted with 1.5 *M* sodium chloride. Analysis of TCA-precipitated proteins by acetic acid–urea PAGE revealed that HMG 14, 17, I and Y were quantitatively eluted in peak 2 (Fig. 1, lane 2), while peak 1 contained HMG 1 and 2 and peak 3 mainly H1. Analysis of each fraction (4 ml) in peak 2 showed that HMG 14 was eluted first followed by HMG 17, I and Y, but none of the proteins was completely separated from the others.

The eluate corresponding to peak 2 was fractionated further by Blue Sepharose chromatography. The eluate was diluted to 0.20 *M* sodium chloride by adding 10 m*M* Tris–HCl (pH 8.0) and applied to the Blue Sepharose column which was equilibrated with the same buffer. The chromatography was carried out with a linear gradient from 0.20 to 0.70 *M* sodium chloride (which elutes HMG 14 and 17), and the remaining proteins step-eluted with 1.5 *M* sodium chloride to avoid the unfavourable dilution of HMG I and Y when these were gradient-eluted (Fig. 3). Acetic acid–urea PAGE of TCA-precipitated proteins revealed that fractions 14–17 contained HMG 17 (Fig. 1, lane 3), fractions 21–24 HMG 14 (Fig. 1, lane 4) and fractions 34–36 a mixture of HMG I and Y (Fig. 1, lane 5). Fractions 18–20 contained a mixture of HMG 14 and 17. These results so far show that HMG I and Y can be completely separated from HMG 14 and 17 by Blue Sepharose chromatography and,

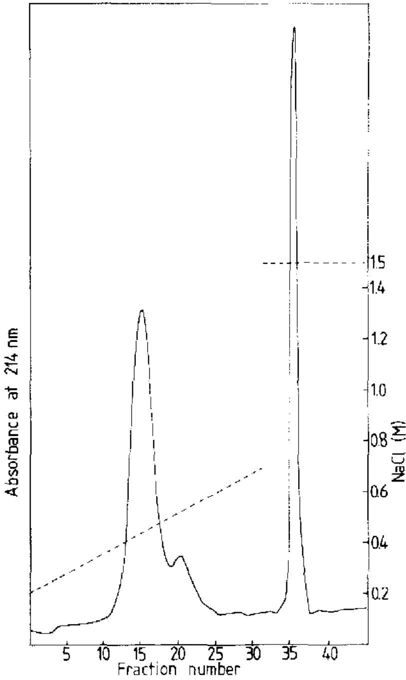

Fig. 3. Chromatography on Blue Sepharose of fractions 23–35 from phosphocellulose chromatography (Fig. 2). The proteins were eluted by increasing the sodium chloride concentration (---------). Absorbance at 214 nm (———).

in addition, pure HMG 17 and 70–90% pure HMG 14 can be obtained. Further purification of HMG I and Y was carried out by HPLC.

Fractionation of HMG proteins by HPLC was carried out with a *n*-propyldimethyl silane, trimethyl-encapped reversed-phase column, using a linear acetoni-

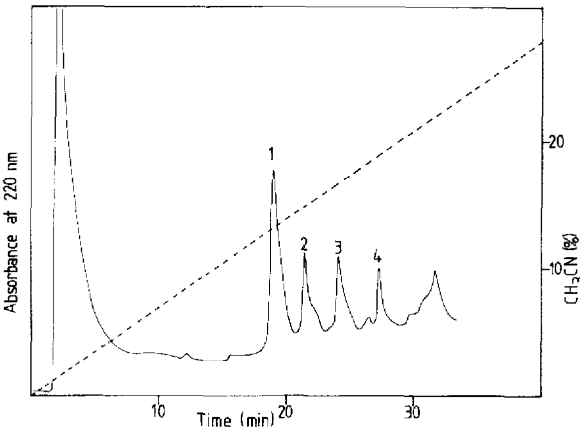

Fig. 4. Reversed-phase HPLC of PCA-soluble proteins from HeLa S3-cells with an Ultrapore RPSC column (75 mm × 4.6 mm). The flow-rate was 1 ml/min. --------, Acetronitrile concentration; ———, absorbance at 220 nm.

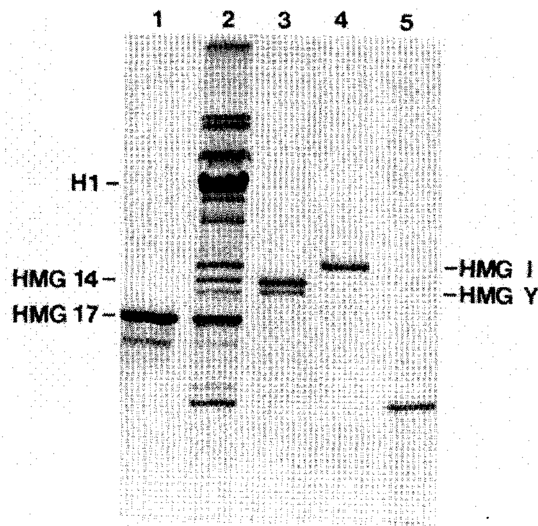

Fig. 5. Acetic acid–urea PAGE of fractions from HPLC (Fig. 4). Lanes: 1 = proteins from peak 1; 2 = PCA-soluble proteins which were applied to the column; 3 = proteins from peak 2; 4 = proteins from peak 3; 5 = proteins from peak 4.

trile gradient. The elution profile obtained with total PCA-soluble proteins from HeLa S3-cells is shown in Fig. 4 (only the first part of the profile is shown). The subsequent gel analysis of some of the peaks (Fig. 5) revealed that HMG I (peak 3, lane 4) was separated from HMG Y (peak 2, lane 3) and was not contaminated by any other proteins. Another protein (peak 4, lane 5) with higher mobility than HMG 17 also seems to be obtained pure from this column. HMG Y and 14 were eluted in the same peak (peak 2, lane 3), which excludes the use of this HPLC column for a single-step purification of these proteins. HMG 17 was slightly contaminated by some unidentified proteins (peak 1, lane 1).

HPLC was also used to fractionate the mixture of HMG I and Y obtained from chromatography on Blue Sepharose. Analysis by acetic acid–urea PAGE revealed that the resulting HMG Y and I preparations were essentially pure (Fig. 1, lanes 6 and 7). From 10^9 HeLa cells, approximately 40 μg HMG I and 10 μg HMG Y were obtained, which is a higher recovery than that obtained by preparative PAGE[3].

There is an alternative procedure for purification of HMG I without using HPLC. When fractions 24–30 from the phosphocellulose column (Fig. 2) were used instead of fractions 24–35, a preparation containing about 80% of the total amount of HMG I and no HMG Y (results not shown) was obtained. HMG I can then be purified to homogeneity by Blue Sepharose chromatography.

DISCUSSION

The low-molecular-weight HMG proteins are relatively abundant chromosomal proteins without enzymatic activity. Their identification has been based on

one- or two-dimensional gel-electrophoretic analysis. HMG 14, 17, I and Y exhibit just minor differences in electrophoretic mobilities, and the interpretation of the gel pattern is further complicated by the fact that there exist species differences in the electrophoretic mobilities, especially with the poorly conserved HMG 14[3]. This means that HMG proteins from different species, exhibiting the same electrophoretic mobility, do not necessarily represent the same or homologous proteins. There has been much confusion concerning the identification of HMG proteins separated by PAGE [9–11]. Accordingly, there is a need for analytical and preparative methods exploiting more subtle properties than molecular mass and charge upon which gel electrophoretic mobilities depend. More subtle differences have already been revealed since these proteins have different affinities for poly(dA · dT) sequences (see Introduction), while the affinity for total DNA is approximately the same.

Blue Sepharose, which contains the dye Cibacron Blue and seems to function as a dinucleotide analogue, has been successfully used for the purification of a variety of chromatin proteins, among them poly(ADP-ribose) polymerase[15–17]. We find that fractionation of the low-molecular-weight HMG proteins on Blue Sepharose completely separates HMG I and Y from HMG 14 and 17 and is a useful step in their identification. Chromatography on Blue Sepharose and subsequent electrophoresis on acetic acid–urea gels enables confirmation of the identity of each individual HMG protein. The considerable difference in the affinity for Blue Sepharose probably reflects fundamental differences in biological function between HMG I and Y on the one hand and HMG 14 and 17 on the other.

In the present work HMG I and Y have been purified by chromatography to homogeneity, as judged by acetic acid-PAGE. The purity is comparable to that obtained by using preparative gel electrophoresis. The advantage of the described chromatographic procedure compared to preparative acetic acid–urea PAGE is the higher recovery; it is also less technically demanding.

HMG I has also been purified in one step by HPLC of PCA-extracted proteins in this work, and previously by Goodwin *et al.*[6] by use of a similar column. In the present work the HMG I isolated seems to be devoid of an additional protein with slightly higher mobility than HMG I, which was present in the preparation of Goodwin *et al.*[6]. However, we found that the high resolution was gradually lost when the HPLC column was repeatedly used to fractionate crude PCA-extracted proteins. If there is a need for highly purified HMG I, preceding purification steps such as chromatography on phosphocellulose and Blue Sepharose should therefore be used in addition to HPLC.

ACKNOWLEDGEMENT

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