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Efficient large-scale purification of non-histone chromosomal proteins HMG1 and HMG2 by using Polybuffer-exchanger PBE94

YOSHIFUMI ADACHI*

Department of Molecular Virology, Institute for Virus Research, Kyoto University, Kyoto 606 (Japan)

SHIGEKI MIZUNO

Department of Agricultural Chemistry, Tohoku University, Sendai 980 (Japan)

and

MICHITERU YOSHIDA

Department of Biological Science and Technology, Science University of Tokyo, Noda 278 (Japan)

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ABSTRACT

A method for the efficient and practical large-scale purification of high-mobility group (HMG) non-histone chromosomal proteins, HMG1 and HMG2, from porcine thymus applying Polybuffer-exchanger PBE94 gel as anion-exchanger has been developed. This method affords higher resolution, purity and yield, than the conventional procedure of CM-Sephadex C-25 ion-exchange column chromatography. Furthermore, use of Polybuffer-exchanger PBE94 column chromatography led to direct preparation of HMG1 and HMG2 from loosely bound non-histone chromosomal protein fraction of chromatin without prefractional precipitation with trichloroacetic acid or prior extraction with perchloric acid. Thus, the application of PBE94 gel as an anion-exchanger to the subfractionation of other kinds of homologous protein is possible.

INTRODUCTION

Non-histone chromosomal proteins in eukaryotic chromatin are known to be implicated in the control of specific gene expression during cell differentiation, proliferation and development. They are an extremely heterogeneous group of proteins with respect to their isoelectric points and molecular sizes. Therefore, it is necessary to purify the various protein components before their intrinsic properties and functions can be studied.

The high-mobility group (HMG) of non-histone chromosomal proteins is a group of relatively abundant chromatin-associated proteins present in the most eukaryotic cells [1–10]. Several procedures have been presented for the purification of four major HMG non-histone chromosomal proteins, HMG1, HMG2, HMG14 and HMG17 [1–12]. In one method, proteins that are loosely bound to

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chromatin were extracted with a low concentration of salt and then fractionally precipitated with 10% trichloroacetic acid (TCA) [1–4,7–11]. The precipitated proteins were then separated by ion-exchange column chromatography using a CM-Sephadex C-25 [1–3,10] or CM-cellulose column [4,8,11]. In another method, part of the protein was extracted from chromatin with 5% perchloric acid (PCA) and then fractionated by CM-Sephadex C-25 column chromatography [6].

As to HMG1 and HMG2, it is necessary to perform rechromatographic procedures, which reduces the yield, because of the highly homologous nature of these proteins [1,3,5,9,12] and of the characteristics of aggregation of their components. In addition, HMG proteins prepared by the above-mentioned methods might lose their intrinsic properties following treatment with strong acid. To overcome these problems, we established an efficient and practical purification method for HMG1 and HMG2, using a Polybuffer-exchanger PBE94 for anion-exchange column chromatography.

EXPERIMENTAL

Materials

Most chemicals were of the purest grade commercially available and were purchased from Wako (Osaka, Japan) or from Sigma (St. Louis, MO, U.S.A.). Polybuffer-exchanger PBE94 was obtained from Pharmacia (Uppsala, Sweden).

Preparation of chromatin from porcine thymus

The preparation of chromatin from porcine thymus was carried out by the method described previously [7], with slight modifications. All operations were performed at 0–4°C. Minced porcine thymus was homogenized in a Waring-blender with six volumes of standard saline citrate (SSC: 0.14 *M* NaCl, 10 m*M* sodium citrate, pH 7.0) containing 1 m*M* phenylmethanesulphonyl fluoride (PMSF) and was then filtered through two layers of gauze. The filtrate was centrifuged at 2000 *g* for 15 min, and the sediment was then washed five times with SSC in the same way. The precipitate was resuspended in 50 m*M* Tris–HCl, 3 m*M* PMSF, pH 7.6 (3 ml/g fresh weight of porcine thymus), and was then centrifuged at 6000 *g* for 10 min. Finally, washing was repeated once more in the same manner.

Extraction of loosely bound non-histone chromosomal proteins

Chromatin was homogenized in 0.35 *M* NaCl, 1 m*M* PMSF with a Potter–Elvehjem PTFE–glass homogenizer and then centrifuged at 5000 *g* for 20 min. Extraction was repeated once more in the same manner. The combined supernatant was then subjected to further fractionation to obtain HMG1 and HMG2.

Purification of HMG1 and HMG2

HMG1 and HMG2 were purified from the loosely bound non-histone chromosomal protein fraction by the following two procedures.

(1) The loosely bound non-histone chromosomal protein fraction was successively fractionated into 2 and 10% TCA-precipitable fractions according to the procedures of Goodwin *et al.* [1]. The 10% TCA-precipitable fraction (50 mg) was dissolved in 3 ml of 10 m*M* Tris–HCl (pH 7.8) and applied to a Polybuffer-exchanger PBE94 column (20 cm x 1.0 cm I.D.), which was pre-activated and pre-equilibrated with 10 m*M* Tris–HCl (pH 7.8). Proteins were then eluted using a 0–1 *M* NaCl linear gradient in the same buffer.

(2) The 0.35 *M* NaCl extract (loosely bound non-histone chromosomal protein fraction) that was not fractionated by TCA precipitation was dialysed overnight against 10 m*M* Tris–HCl (pH 7.8), followed by centrifugation at 5000 *g* for 20 min to remove any debris. The dialysate (60 mg of protein) was then subjected to PBE94 column chromatography as described above.

Determination of protein and nucleic acid concentrations

The protein concentration was determined by the method of Lowry *et al.* [13] using bovine serum albumin as the standard, or else by the absorbance at 280 nm. The nucleic acid concentration was determined according to the method of Schneider [14].

Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [15]. The gels were stained with Coomassie brilliant blue R250.

Amino acid analysis

Protein was hydrolysed in 6 *M* HCl at 110°C for 24 h in an evacuated tube. The hydrolysate was then analysed using a Hitachi 835 amino acid analyser. The amino acid composition was expressed as mol per 100 mol of the total amino acid content.

RESULTS AND DISCUSSION

The 10% TCA-precipitable fraction of the loosely bound non-histone chromosomal proteins obtained from porcine thymus was subjected to CM-Sephadex C-25 ion-exchange chromatography according to the method of Goodwin *et al.* [1]. The resolution of both HMG1 and HMG2 by CM-Sephadex C-25 chromatography was found to be inadequate (data not shown). Therefore, to obtain a better resolution and yield, we investigated improvements to the chromatography step. Polybuffer-exchanger PBE94 is an anion-exchange gel originally developed for chromatofocusing with Polybuffer or Pharmalyte 8–10.5. PBE94 is composed of Sepharose CL-6B coupled with a variety of dissociation radicals by ether-linking and has an even and high capacity over a wide pH range (3–11) [16]. For these reasons, PBE94 was used for anion-exchange chromatography in the separation of HMG1 and HMG2.

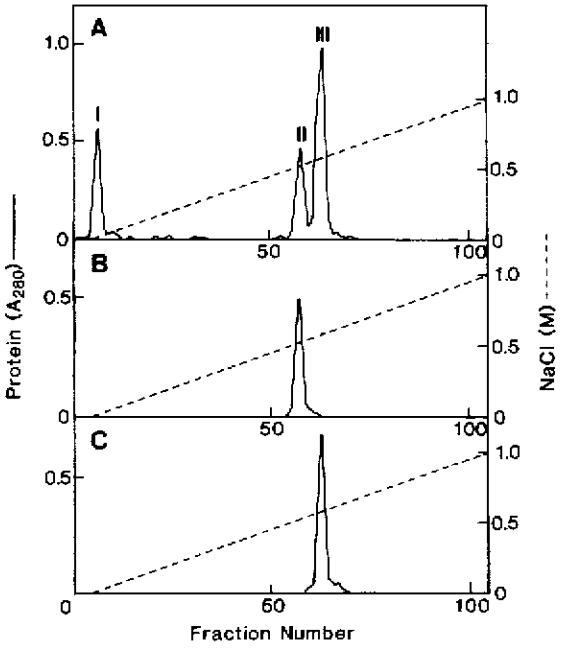

Fig. 1. Purification of HMG1 and HMG2 by PBE94 column chromatography. (A) The 10% TCA-precipitable fraction (50 mg of protein) in 3 ml of 10 m*M* Tris–HCl (pH 7.8) was applied to a PBE94 column (20 cm x 1.0 cm I.D.). Proteins were eluted by a 0–1 *M* NaCl linear gradient in the same buffer. The flow-rate was 10 ml/h, and fractions of 4 ml were collected. (B) Peak II was rechromatographed under the same conditions. (C) Peak III was rechromatographed under the same conditions.

Fig. 1A shows the elution profile of the 10% TCA-precipitable fraction on the PBE94 column chromatography. The proteins were found to be fractionated into three peaks (I–III) by NaCl linear gradient elution. SDS-PAGE showed that peak I contained HMG14, HMG17 and an unidentified component similar in mobility to the A24 protein (histone uH2A) (Fig. 2, lane 1). Peak II was assigned to HMG2 and peak III to HMG1, on the basis of their immunoreactivities for anti-HMG2 and anti-HMG1 antibodies (data not shown) and their respective molecular masses of 25 000 and 26 000 dalton (Fig. 2, lanes 2 and 3). When the separated HMG2 (peak II) and HMG1 (peak III) proteins were rechromatographed independently under the same conditions, each protein was eluted sharply and reproducibly at the same NaCl concentrations as before (Figs. 1B and C). The HMG1 and HMG2 proteins separated by this one-step column chromatography method (Fig. 1A) possessed a high purity (greater than 98%) judging from the densitometric analysis of the gels from SDS-PAGE. The recovery of each protein by this method was *ca.* 90%. The amino acid compositions of the HMG1 and HMG2 purified from porcine thymus (Table I) were found to be identical with those previously reported from various sources [3,10,17]. Thus,

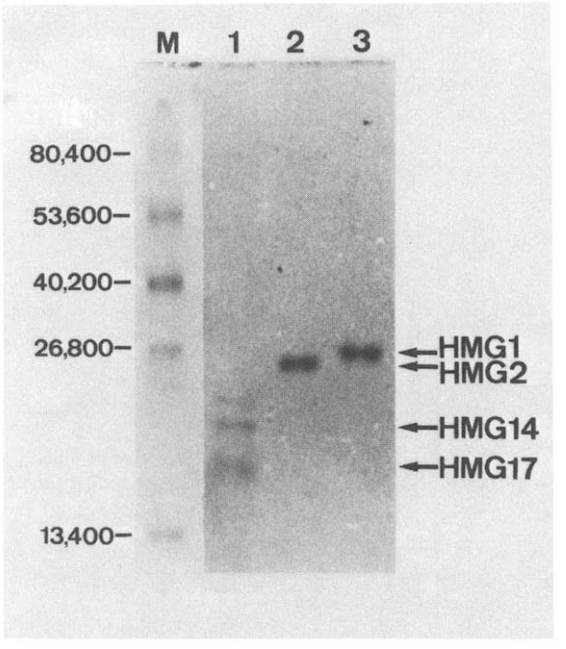

Fig. 2. SDS-PAGE of the peaks obtained by PBE94 column chromatography from the 10% TCA-precipitable fraction. Polyacrylamide gel (12.5%) with 0.1% SDS was used. After electrophoresis, the gel was stained with Coomassie brilliant blue R250. Lane M, marker proteins; lane 1, peak I; lane 2, peak II; lane 3, peak III.

PBE94 column chromatography was found to be applicable to the isolation of HMG1 and HMG2.

We also attempted to isolate HMG1 and HMG2 directly from the loosely bound non-histone chromosomal protein fraction (0.35 *M* NaCl extract) without pre-fractional precipitation with 10% TCA. The 0.35 *M* NaCl extract was fractionated into twelve fractions (fractions A–L) by PBE94 column chromatography, as shown in Fig. 3A. Fig. 4 shows the results of SDS-PAGE for each fraction. Fractions A and B comprised mainly HMG14 and HMG17 (Fig. 4, lanes 1 and 2) and were eluted at almost identical NaCl concentrations as shown in Fig. 1A. Fractions D–J contained heterogeneous non-histone chromosomal proteins (Fig. 4, lanes 4–10). The recovery of these fractions from the column varied because of solubility problems caused by protein aggregation. Fractions K and L were eluted from the column by 0.56 and 0.62 *M* NaCl, respectively. These elution concentrations approximated those required to elute HMG2 (0.54 *M* NaCl in Fig. 1A) and HMG1 (0.60 *M* NaCl). Fraction K contained a single protein band of 25 000 dalton, and the protein in fraction L formed a single band at 26 000 dalton on electrophoresis (Fig. 4, lanes 11 and 12). On the basis of the NaCl concentrations required for elution, the molecular masses, and the immunoreactivities (data not shown), the main components in fractions K and L were consid-

TABLE I

COMPARISON OF AMINO ACID COMPOSITIONS OF HMG1 AND HMG2 FROM PORCINE THYMUS, CALF THYMUS AND DUCK ERYTHROCYTES

The values for each amino acid are expressed as mole percent.

Amino acid	Porcine thymus		Calf thymus[a]		Duck erythrocytes[a]
	HMG1	HMG2	HMG1	HMG2	HMG1
Asp	10.9	9.8	11.0	9.6	11.6
Thr	2.3	1.9	3.0	2.7	2.1
Ser	5.0	7.6	5.3	7.5	4.6
Glu	18.9	18.4	17.8	17.1	20.0
Pro	6.2	7.4	5.2	7.7	5.5
Gly	5.4	7.0	5.2	6.3	5.4
Ala	9.2	7.3	8.2	7.3	7.7
Val	2.3	1.5	2.3	2.1	3.4
Cys	0.7	0.8	3.0	1.6	N.D.[b]
Met	2.0	2.3	1.1	1.2	1.4
Ile	1.9	1.5	1.9	1.4	1.8
Leu	2.0	2.1	2.2	2.1	2.9
Tyr	2.3	2.8	2.8	2.6	2.5
Phe	4.1	3.8	5.6	4.2	4.5
Lys	20.3	19.4	16.8	19.1	21.4
His	1.7	1.9	1.7	1.7	1.4
Arg	3.8	4.4	5.5	5.1	3.7
Acidic amino acid (A)	29.9	28.2	28.8	26.7	31.6
Basic amino acid (B)	25.8	25.7	24.0	25.9	26.5
A/B ratio	1.16	1.10	1.20	1.03	1.19

[a]These data are quoted from refs. 3 and 10.
[b]N.D., not detected.

ered to be HMG2 and HMG1, respectively. Rechromatography of fractions K and L under the same conditions showed identical elution profiles for HMG2 and HMG1 with good reproducibility (Fig. 3B and C). Fraction M contained detectable amounts of low-molecular-mass RNA (less than 5S). Thus, it was possible to isolate HMG1 and HMG2 directly from the 0.35 *M* NaCl extract by PBE94 column chromatography without the need for pre-fractional precipitation using 10% TCA or prior extraction with 5% PCA. Moreover, the isolated HMG1 and HMG2 maintained their *in vitro* DNA unwinding activity [18].

This procedure appears to be efficient and practical for the large-scale purification of HMG1 and HMG2. It provides a higher resolution, purity and yield than the conventional method of CM-Sephadex C-25 ion-exchange column chromatography [1–3,10]. We were able to prepare 100 mg of HMG1 and 60 mg of HMG2 from 3 kg of porcine thymus by 10% TCA precipitation followed by PBE94 column chromatography. Furthermore, we could also purify 4.1 mg of HMG1

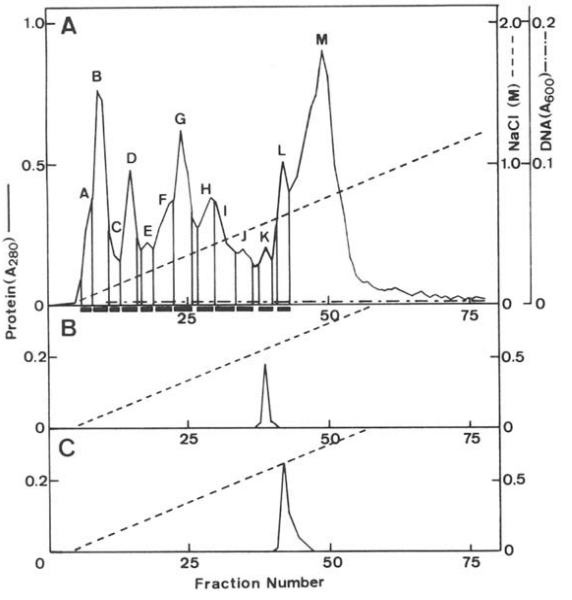

Fig. 3. PBE94 column chromatography of the loosely bound non-histone chromosomal protein fraction (0.35 *M* NaCl extract from chromatin). (A) The 0.35*M* NaCl extract (60 mg of protein) was applied to a PBE94 column (20 cm x 1.0 cm I.D.) without pre-fractional 10% TCA precipitation. The proteins were eluted by a 0–1.5 *M* NaCl linear gradient in 10 m*M* Tris·HCl (pH 7.8). The flow-rate was 10 ml/h, and fractions of 4 ml were collected. The protein fractions marked by thick bars were combined and named as indicated. (B) Peak K was rechromatographed under the same conditions. (C) Peak L was rechromatographed under the same conditons.

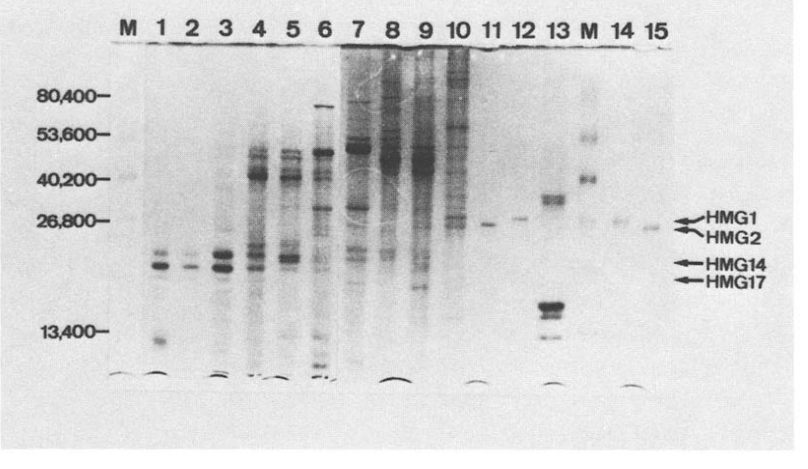

Fig. 4. SDS-PAGE of the fractions obtained by PBE94 column chromatography from the loosely bound non-histone chromosomal protein fraction (0.35 *M* NaCl extract from chromatin). Polyacrylamide gel (12.5%) with 0.1% SDS was used. After electrophoresis, the gel was stained with Coomassie brilliant blue R250. Lanes 1–12 correspond to fractions A–L in Fig. 3. Lane M, marker proteins; lane 13, whole histone fraction of porcine thymus; lane 14, purified HMG1; lane 15, purified HMG2.

and 3.3 mg of HMG2 directly from the 0.35 *M* NaCl extract (160 mg of protein) by one-step PBE94 column chromatography, avoiding exposure of the proteins to extremely acidic conditions. HMG1 and HMG2 obtained under these milder conditions are desirable for studies of their functions and intrinsic properties, especially the interactions of these proteins with DNA and the nucleosome. Finally, the PBE94 column should be applicable to the fractionation of various other kinds of non-histone chromosomal protein.

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REFERENCES

1 G. H. Goodwin, R. H. Nicolas and E. W. Johns, *Biochim. Biophys. Acta*, 405 (1975) 280.
2 G. H. Goodwin, L. Woodhead and E. W. Johns, *FEBS Lett.*, 73 (1977) 85.
3 R. Sterner, L. C. Boffa and G. Vidali, *J. Biol. Chem.*, 253 (1978) 3830.
4 D. C. Watson, E. H. Peters and G. H. Dixon, *Eur. J. Biochem.*, 74 (1977) 53.
5 C. Bonne, P. Sautiere, M. Duguet and A. M. Recondo, *J. Biol. Chem.*, 257 (1982) 2722.
6 J. M. Walker and E. W. Johns, *Biochem. J.*, 185 (1980) 383.
7 M. Yoshida, Y. Ohtaki, T. Fujita and K. Shimura, *J. Biochem.*, 88 (1980) 425.
8 M. Yoshida and K. Shimura, *J. Biochem.*, 95 (1984) 117.
9 J. M. Walker, K. Gooderham, J. R. B. Hastings, E. Mayes and E. W. Johns, *FEBS Lett.*, 122 (1980) 264.
10 A. Rabbani, G. H. Goodwin and E. W. Johns, *Biochem. J.*, 173 (1978) 497.
11 C. S. Teng, K. Gallagher and C.T. Teng, *Biochem. J.*, 176 (1978) 1003.
12 J. M. Walker, in E. W. Johns (Editor), *The HMG Chromosomal Proteins*, Academic Press, London, 1982, p. 69.
13 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
14 W. C. Schneider, *J. Biol. Chem.*, 161 (1945) 293.
15 U. K. Laemmli, *Nature*, 227 (1970) 680.
16 *Chromatofocusing with Polybuffer and PBE*, Pharmacia, 1986.
17 K. Tsuda, M. Kikuchi, K. Mori, S. Waga and M. Yoshida, *Biochemistry*, 27 (1988) 6159.
18 M. Yoshida, *J. Biochem.*, 101 (1987) 175.