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Short communication

Use of reversed-phase high-performance liquid chromatography on polystyrene–divinylbenzene columns for the rapid separation and purification of acid-soluble nuclear proteins

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Abstract

The suitability of polystyrene–divinylbenzene reversed-phase HPLC columns for rapid separation and purification of acid-soluble nuclear proteins was evaluated. We used a polystyrene–divinylbenzene reversed-phase HPLC column (PLRP-S) for purification of nuclear proteins extracted with 0.3 *M* HCl or 5% $HClO_4$. We are able to obtain electrophoretically pure fractions for a number of nuclear proteins including HMG14, HMG17 and variants of histone H3. The identity of proteins in these fractions was confirmed by immunochemical analysis, protein sequencing, mass spectrometry and migration on two-dimensional polyacrylamide gel electrophoresis. These methods do not require special preparation of the sample and are quicker than similar published methods.

Keywords: Stationary phases, LC; Proteins; Histone H3; HMG14; HMG17

1. Introduction

Reversed-phase liquid chromatography (RPLC) is becoming the most popular mode of HPLC for peptide and protein purification due to its superior resolving power. The great potential of this method, however is accompanied by some drawbacks and technical problems. For example the classical silica-based reversed-phase columns are unusable with aggressive denaturing agents, extreme pH and elevated temperatures. In fact silica-based matrices will dissolve above pH 7.5 [1].

With the introduction of polystyrene–divinylbenzene packings, biomolecules in more aggressive buffers can be separated rapidly with high resolution. Such matrices are capable of withstanding denaturing agents, organic eluents and high temperatures, which are all used to enhance resolution. In addition they are chemically stable through the pH range 1–13, so extending the range of reversed-phase chromatography beyond that previously possible with silica-based packings (pH 2–7).

The objectives of this work were to establish a rapid method for purification of nuclear proteins involved in signal transduction (histone H3, HMG etc.) that is compatible with the extraction procedures for these proteins which conventionally involve the use of acids (0.3 *M* HCl, 5% perchloric acid etc.). Although remarkable results for separating histones [2,3] and HMG [4] have been obtained by using silica-based reversed-phase columns, samples require special preparation before the separation.

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We used a polystyrene–divinylbenzene reversed-phase HPLC column (PLRP-S) for purification of nuclear proteins extracted with 0.3 *M* HCl or 5% $HClO_4$ where the extracts were injected directly into the column. We are able to obtain electrophoretically pure fractions for a number of nuclear proteins including HMG14, HMG17 and histone H3 variants.

2. Experimental

2.1. Cell culture and extraction of C3H 10T1/2 cells

Mouse C3H 10T1/2 fibroblasts were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal calf serum. Medium was aspirated and cells were lysed directly in cold Triton-deoxycholate (DOC) buffer as described in [5]. Following pelleting (microcentrifuge, 15 min) the Triton-DOC-insoluble material, primarily cytoskeletal and nuclear components, was treated with either 0.3 *M* HCl or 5% perchloric acid and the soluble proteins analyzed directly by HPLC.

2.2. HPLC

All experiments were performed on a Gynkotek HPLC using two HPLC pumps (Models 300 and 480). The eluted fractions were detected using a diode array detector Model UVD-320S. The detection signal was analyzed using Gynkosoft Version 5.21C software.

2.3. Columns

An Eurosil Bioselect 300 column (250×4.6 mm I.D.) was obtained from Knauer and a PLRP-S 300 Å column 250×4.6 mm I.D.) from Polymer Laboratories. Source 15RPC chromatography media based on polystyrene–divinylbenzene beads of 15 μm were obtained from Pharmacia Biotech and packed in our laboratory as a preparative 10-ml column.

2.4. Chemicals

Acetonitrile Far UV, trifluoracetic acid (TFA, sequencing grade), hydrochloric acid and perchloric acid were obtained from Fisons. Water was purified using a Elgastat Maxima apparatus. All mobile phases were degassed before use with helium sparking. During chromatography, the mobile phases were degassed continuously by passage through a Shodex KT-27 degasser.

2.5. HPLC separation

All separations were performed at room temperature. Detailed descriptions of the mobile phases and gradients used are given below in Section 3.

2.6. Gel electrophoresis

Acetic acid–urea polyacrylamide gel electrophoresis (PAGE) was performed as described in [5].

2.7. Protein microsequencing

Proteins were prepared for sequencing as described earlier [6]. Proteins and peptides were sequenced using an Applied Biosystem automated sequencer.

2.8. Quantitation of protein

Protein concentration was estimated using the Coomassie blue (Bradford) protein assay [7].

3. Results

We first tested the suitability of a column PLRP-S column for purification of nuclear proteins extracted with 0.3 *M* HCl. Extracts were prepared as described in Section 2. 10 mg cells (wet mass) were extracted with 300 μl 0.3 *M* HCl. This extract contains predominantly histones as described earlier [8]. A 100-μl volume of this extract containing approximately 0.2 mg of protein was diluted in 0.1% TFA, applied to a 250×4.6 mm I.D. PLRP-S 300 Å column and eluted at 1 ml/min with a 60-min linear gradient (Fig. 1A). The elution profile was comparable with those obtained with a silica-based Eurosil Bioselect 300 column (not shown). The major peaks were analyzed by acetic acid–urea PAGE (Fig. 1B).

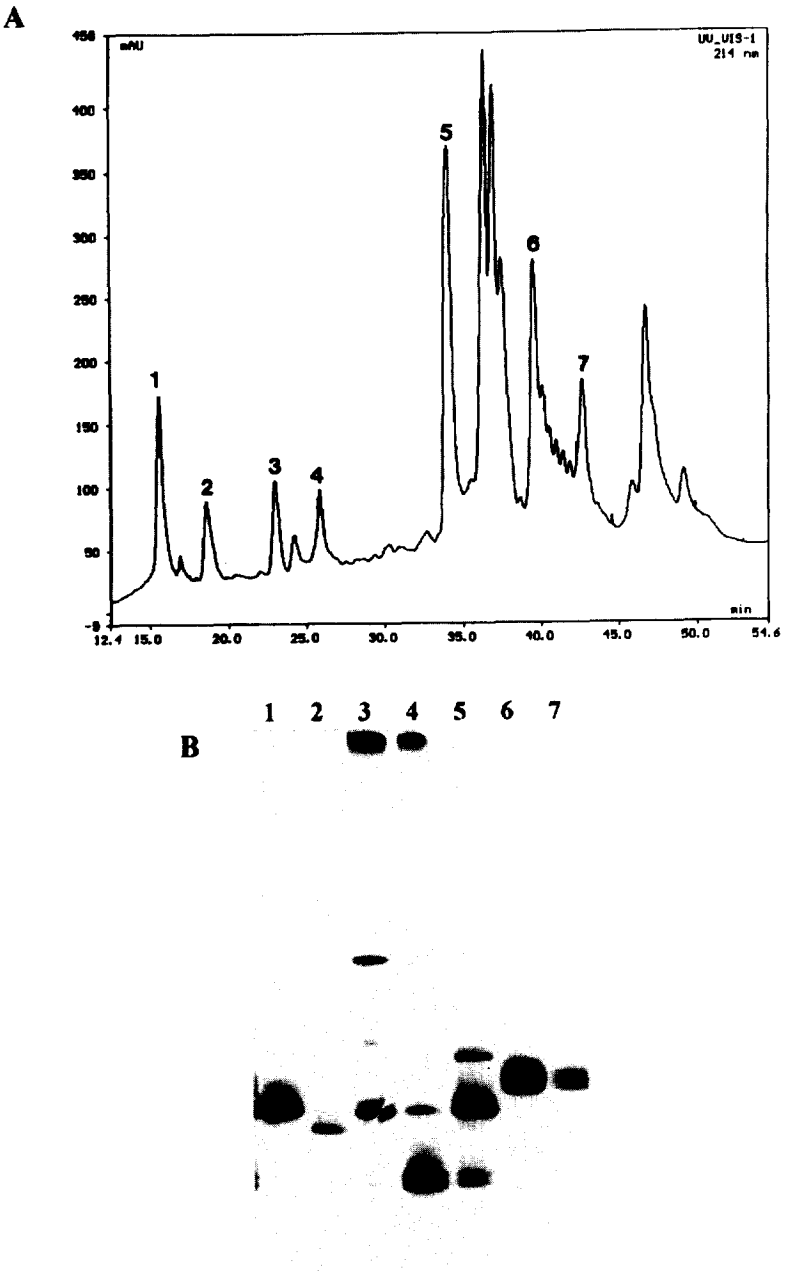

Fig. 1. (A) Separation of HCl extractable nuclear proteins by reversed-phase chromatography using PLRP-S 300 Å column. Nuclei were extracted with 0.3 *M* HCl, extracts were diluted 1:2 in 0.1% TFA and directly injected into the column and eluted with a gradient from 0 to 70% acetonitrile. 0.1% TFA was present in buffer A and B. (B) Proteins from the eight peaks indicated as 1–8 in panel A were analyzed on acetic acid–urea PAGE. Peak 6 contains histone H3.2 and its acetylated modifications and peak 7 contains histone H3.1 and its acetylated modifications.

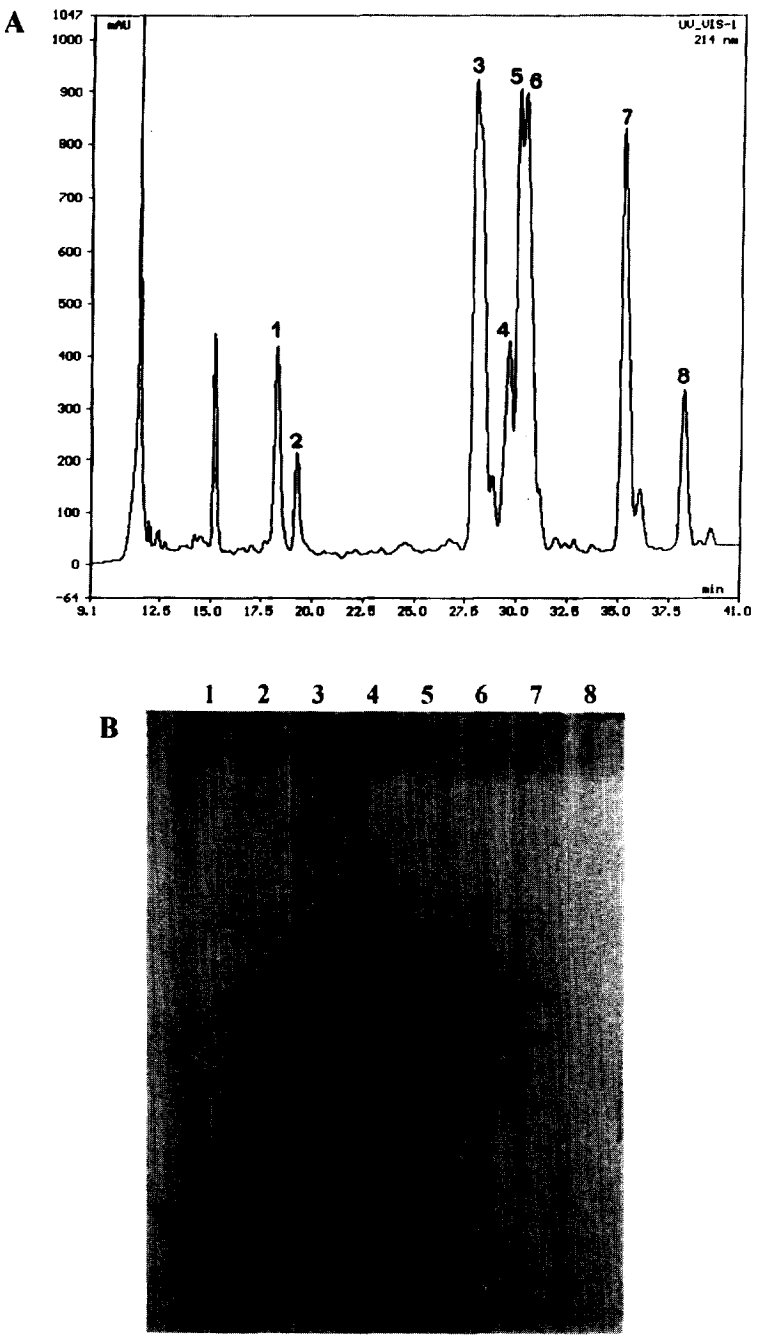

Fig. 2. (A) Separation of perchloric acid extractable nuclear proteins by reversed-phase chromatography using a polystyrene–divinylbenzene reversed-phase column-PLRP-S 300 Å. Nuclei were extracted with 5% PCA, extracts diluted 1:2 in 0.1% TFA and directly injected into the column and eluted with a gradient from 0 to 30% acetonitrile. 0.1% TFA was present in buffer A and B. (B) Peaks were analyzed on acetic acid–urea PAGE. Peak 1 consists of HMG17 and peak 2 contains HMG14 and its phosphorylated modification.

Further analysis of the proteins in peaks 6 and 7 by protein sequencing, mass spectrometry and migration on two-dimensional PAGE (not shown) revealed that these two peaks consist of practically pure proteins: the variants of mouse H3–H3.2 and H3.1 correspondingly and their acetylated modifications. We were able to recover approximately 50 μg H3.2 and 30 μg H3.1 per run.

In addition we evaluated use of the same column for purification of PCA extractable nuclear proteins. Extracts were prepared as described in Section 2. 50 mg cells (wet mass) were extracted with 300 μl 0.3 *M* HCl. This extract contains predominantly HMG proteins and histone H1 as described earlier [9]. A 100-μl volume of this extract containing approximately 0.1 mg of protein was diluted in 0.1% TFA up to 1 ml, applied to the column and eluted at 1 ml/min with a 40-min linear gradient (Fig. 2A). Analysis of the major peaks by acetic acid–urea PAGE demonstrated that at least two of them (No. 1 and 2) contain pure proteins (Fig. 2B). Using protein sequencing, mass spectrometry, migration on two-dimensional PAGE and an antibody against C-terminus of mouse HMG14, we were able to identify the protein in peak 1 as HMG14, and the protein in peak 2 as HMG17. We were able to recover approximately 25 μg HMG14 and 20 μg HMG17 per run.

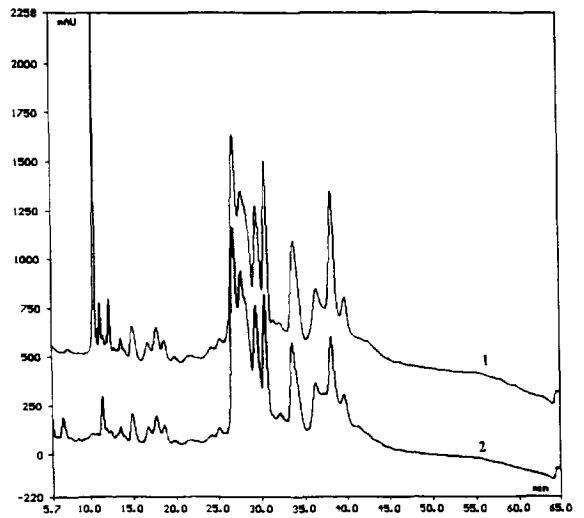

Fig. 3. (1) Stability and reproducibility of polystyrene–divinylbenzene media. Nuclei were extracted with 0.3 *M* HCl, extracts diluted 1:2 in 0.1% TFA and directly injected into a preparative 10-ml column packed in our laboratory and eluted with a gradient from 0 to 70% acetonitrile. 0.1% TFA was present in buffer A and B. (2) Chromatography of the same sample after 20 runs.

Finally we carried out an experiment to check the stability of the polystyrene–divinylbenzene package and the reproducibility of the chromatograms after long acid treatment of the column. We used routinely a preparative 10-ml reversed-phase column packed in our laboratory with Source 15RPC chromatography media based on polystyrene–divinylbenzene beads for purification of H3 from HCl extracts as described above. We were able to carry out twenty identical runs on this column (Fig. 3), whereas the separation deteriorated dramatically after five runs when we separated HCl extracts on a silica-based C_{18} column (Eurosil Bioselect 300) (not shown).

4. Discussion

These experiments demonstrate the suitability of polystyrene–divinylbenzene reversed-phase HPLC columns for rapid separation and purification of acid-soluble nuclear proteins. Due to the high chemical stability of this column package a direct injection of the acid extract is possible, thus reducing the time and increasing the yield and the reproducibility.

We describe methods for purification of nuclear proteins that are rapid, do not require a special procedure for preparation of the sample and result in electrophoretically pure fractions of several nuclear proteins namely: H3.1, H3.2, HMG14 and HMG17. As it has been demonstrated that the biological activity of both HMG proteins [10] and histones [11] can be recovered after acid extraction, the purified proteins can be used for analytical and some functional experiments.

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