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Separation and Purification of Multiply Acetylated Proteins Using Cation-Exchange Chromatography

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Abstract

High-performance liquid chromatography (HPLC) is extremely useful for the study of proteins and the characterization of their posttranslational modifications. Here we describe a method that utilizes cation-exchange HPLC to separate multiply acetylated histone H3 species on the basis of their charge and hydrophilicity. This high-resolution method allows for the separation of histone H3 species that differ by as few as one acetyl group, and is compatible with subsequent analysis by a variety of techniques, including mass spectrometry and western blotting.

Keywords

Cation-exchange chromatography; PolyCAT A; Acetylation; HPLC; Histone H3; Acetic anhydride

1. Introduction

Dynamic protein acetylation is known to impart key functional changes in biological systems, in particular, acetylation of eukaryotic histone proteins helps govern transcriptional competency of the associated DNA. It is increasingly appreciated that acetylation can reside at multiple sites on the same histone polypeptide, and can even occur together with other small covalent posttranslational modifications (PTMs) like methylation and phosphorylation. Specific combinations of such PTMs likely “code” for a variety of integrative chromatin-templated processes. However, the mechanistic basis of this regulation is poorly understood, in part because detection of multiple PTM states is complicated. For example, antibody-based recognition of PTMs is often dependent on epitope accessibility, which can vary depending on the modification state of surrounding residues. Fortunately, mass spectrometry methodologies have recently emerged that permit relatively unbiased PTM analysis on long stretches of amino acids or even intact proteins, although the complexity of PTM states usually necessitates extensive front-end purification to be suitable for such approaches (1, 2).

While gel-based systems including acid–urea gel electrophoresis can be used to resolve acetylated (and phosphorylated) protein isoforms, the amount of material purified is relatively small and is in a form that is not compatible with limited proteolytic treatment necessary to utilize the mass spectrometry approaches referred to above. These deficiencies are remedied by cation-exchange HPLC. Under certain conditions, cation-exchange columns, like polyCAT A, permit separation of protein isoforms by exploiting their hydrophilic and electrostatic differences (3–5). For example, protein acetylation decreases positive charge and hydrophilicity, which contribute to the superior resolving power of polyCAT A chromatography. Furthermore, this chromatographic approach can also be

performed on a preparative scale, yielding amounts of protein that are compatible with subsequent analysis by mass spectrometry and western blotting (6).

Below we describe our approach to separate and purify distinctly acetylated isoforms of histone H3 from the ciliated protozoan *Tetrahymena thermophila* by using polyCAT A-based chromatography. We start with highly purified histone H3 from *Tetrahymena* which contains a complex mixture of acetylation states ranging from no acetylation to five (and possibly more) acetylated lysines. We treat this purified H3 with acetic anhydride (Ac_2O) to generate hyperacetylated isoforms in vitro. The hyperacetylated and endogenous H3 samples are independently applied to the polyCAT A column and eluted over a steep salt gradient. The concentrations of salt required to elute hyper- and endogenously acetylated H3 isoforms are used to demarcate elution conditions for all possible acetylated H3 isoforms. Endogenous H3 is then reapplied to the column and eluted using the truncated range of salt concentrations and an extended run time, thus achieving maximal resolution among acetyl isoforms. Lastly, samples are desalted by reversed phase-high-performance liquid chromatography (HPLC) (RP-HPLC) and stored for subsequent analysis by mass spectrometry and western blots. Although we resolve histone H3 acetyl states in this protocol, this methodology can be adapted for many other acetylated proteins as well.

2. Materials

All commercially available solutions and reagents should be of analytical grade. Water used to prepare solutions should be ultra-pure and highly deionized. Additionally, any solution made for HPLC should be purified using at least a 0.45- μm filter and degassed prior to injection into the column.

2.1. In Vitro Acetylation of Proteins

1. Acetic anhydride (Ac_2O).
2. Methanol (MeOH).
3. 2 $\times$ Reaction buffer: 100 mM ammonium bicarbonate (NH_4HCO_3 , m.w. = 79.05). Dissolve 79 mg in 10 mL of water and do not adjust the pH (see Note 1).
4. RP-HPLC-purified protein dissolved in water (~2 mg/mL) (see Note 2).
5. Microcentrifuge tubes.
6. Speed-vac centrifuge.

2.2. Desalting by Reversed Phase-HPLC

1. HPLC pump and fraction collector.
2. C8 column (220 $\times$ 4.6 mm, RP-300, #0711-0059, Perkin Elmer, Waltham, MA, USA).
3. Acetonitrile (CH_3CN), HPLC grade.
4. Trifluoroacetic acid (TFA).
5. Graduated cylinders, 1 and 100 mL.

¹Due to the volatility of ammonium bicarbonate we recommend making fresh solution each time before use.

²We obtain purified histone H3 by following the nuclear preparation, acid extraction, and RP-HPLC purification protocols described in Shechter et al. (7). During nuclear preparation, we try to preserve endogenous protein acetylation by including 10 mM butyric acid, an HDAC inhibitor, in buffers.

6. Magnetic stirrer, stir bars, and 1 L suction flasks.
7. Kontes vacuum filtration assembly (#XX1504700, Fisher Scientific, Fair Lawn, NJ, USA).
8. Durapore PVDF membrane filters 0.45 μm (#HVLP047000, Millipore, Billerica, MA, USA).
9. 1 mL Hamilton syringe equipped with blunt-end needle point.

2.3. PolyCAT A Chromatography Buffers

1. Phosphate buffer: Stock solution is made by mixing appropriate amounts of NaH_2PO_4 (sodium phosphate monobasic, solution 1) and Na_2HPO_4 (sodium phosphate dibasic, solution 2) to obtain the final buffer at pH 7. Start by making 1 M stocks of each solution. Dissolve 6.9 g of monobasic $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (m.w. = 138) in water to a final volume of 50 mL (solution 1) and 7.1 g of dibasic Na_2HPO_4 (m.w. = 142) in water to a final volume of 50 mL (solution 2). Shake both solutions vigorously to help dissolve. While stirring 50 mL of solution 2, adjust to pH 7 by slowly (1 mL at a time) adding solution 1. Store the 1 M phosphate buffer, pH 7 at room temperature.
2. Urea ($\text{CO}(\text{NH}_2)_2$).
3. Dithiothreitol (DTT): 1 M solution dissolved in water (see Note 3).
4. Sodium chloride (NaCl).
5. Bio-Rex MSZ 501 (D) deionizing resin (#142-7425, BioRAD, Hercules, CA, USA).
6. Gravity column (#732-1010, BioRAD, Hercules, CA, USA).
7. Two 500 mL graduated cylinders.
8. Magnetic stirrer, stir bar, and 1 L flask.

2.4. PolyCAT A Column Chromatography Components

1. HPLC pump and fraction collector.
2. PolyCAT A column (200×4.6 mm, 5 μm , 1,000 Å, PolyLC, Columbia, MD, USA) (see Notes 4 and ⁵).
3. 1 mL Hamilton syringe equipped with blunt-end needle point.
4. Dot blot apparatus (#170-6545, BioRAD, Hercules, CA, USA).
5. PVDF membrane.
6. Necessary reagents and instructions for immunodetection are described in ref. 6.

³To make 1 M stock solution, dissolve 1.54 g of DTT (m.w. = 154.25) in water to a final volume of 10 mL. Filter, divide to 1 mL aliquots, and store in -20°C .

⁴The specifications of the column will vary depending on the mass and properties of the protein being studied. Contact the manufacturer for additional help with selecting the proper column.

⁵PolyCAT A column performance is significantly enhanced when prewashed with 40 mM EDTA-2Na overnight using a slow flow rate (0.25 mL/min). Do not adjust the pH of this solution and remember to filter purify the solution prior to passing it through the column. After the EDTA prewash, wash the column with 10 mL filtered water and proceed to equilibration with "polyCAT solution A."

3. Methods

3.1. In Vitro Acetylation of Proteins

1. Make 50 μL of acetylation buffer by mixing 12.5 μL of acetic anhydride with 37.5 μL of methanol (see Note 6).
2. In a separate microcentrifuge tube, mix 10 μL 2 $\times$ reaction buffer and 10 μL of protein (2 mg/mL) (see Note 7).
3. Combine the two solutions (70 μL final volume) and briefly mix by gentle tapping.
4. Incubate at room temperature for 30 min.
5. Stop the reaction by drying with a speed-vac centrifuge.
6. Store dried samples at -20°C .
7. The extent of acetylation can be tested by mass spectrometry, immunoblot assays, or acid-urea gels after desalting the samples following the procedures in Subheading 3.2 (Fig. 1 and see Note 8).

3.2. Desalting by Reversed Phase-HPLC

1. Prepare mobile phase for RP-HPLC: 1 L solution A—5% acetonitrile, 95% water, and 0.1% TFA; and 1 L solution B—90% acetonitrile, 10% water, and 0.1% TFA (see Note 9).
2. Vacuum filter both chromatography solutions using a 0.45 μm PVDF filter (see Note 10).
3. Transfer solutions into clean 1 L suction flasks with stir bar and stir at medium speed for 10 min under vacuum to degas each buffer.
4. Equilibrate the C8 column with 10 mL of solution A at a flow rate of 0.8 mL/min.
5. Take dried samples from Subheading 3.1 and resuspend in 200 μL of solution A by vortexing.
6. Spin down $>15,000 \times g$ for 5 min to pellet any particulates.
7. Load sample in Hamilton syringe, inject into the HPLC machine, and run the chromatography method at 0.8 mL/min according to Table 1.
8. Record absorbance at 214 nm and collect relevant fractions (see Note 11).
9. Dry down fractions by speed-vac centrifugation.
10. Once dried, the samples can be stored at -20°C .

⁶The in vitro acetylation protocol was adapted from ref. 8.

⁷This procedure is used to acetylate 20 μg of protein. If more acetylated protein is desired adjust the volume of the final reaction as needed and keep the same ratio between the acetylation and reaction buffers.

⁸In addition to acetylating lysines, this treatment also yields acetylated N-termini.

⁹Prepare appropriate volumes of acetonitrile and water in separate graduated cylinders prior to mixing. Upon mixing the actual volume (~980 mL) will be less than the expected volume (1,000 mL) due to entropy changes. Carefully add TFA in the end and proceed to filtration.

¹⁰This critical step is required for all solutions used in HPLC. Removal of particulates extends the life of the column and minimizes clogging during a run.

¹¹In general, acetylation or other small PTMs do not dramatically affect the column retention time of proteins, so hypoacetylated proteins will elute in the same fractions as hyperacetylated proteins using RP-HPLC. If the elution time of the sample is not known, collect fractions over the entirety of the initial run. After determining the elution time of the sample, the fraction collection window can be narrowed.

3.3. Preparation of the Mobile Phase for PolyCAT Chromatography

These solutions are made fresh before use and discarded at the end of each day. A flowchart for the procedures in this section and the final concentration of the components for the mobile phases are provided in Fig. 2.

1. In a 1 L flask, dissolve 192.2 g of urea (m.w. = 60.06) in 230 mL of water. Stir at room temperature until the urea completely dissolves (see Note 12).
2. Remove ions from the urea solution by adding 1–5 g of deionizing resin. Deionize for 1 h while stirring. Add more resin as needed (see Note 13).
3. Remove the resin by passing the urea solution through a gravity column. Collect the flow through in a 500 mL graduated cylinder.
4. While stirring on a magnetic plate add 9.2 mL of 1 M phosphate buffer to the flow through.
5. Add 200 μ L of 1 M DTT.
6. Adjust the volume to 380 mL with water.
7. Split the solution evenly into two graduated cylinders (190 mL in each cylinder). In the first cylinder add 10 mL water and label this “solution A” for polyCAT chromatography.
8. While stirring the second cylinder dissolve 11.68 g of NaCl (m.w. = 58.44) to obtain a final concentration of 1 M NaCl and label this as solution “B” for polyCAT chromatography. Adjust the final volume of solution B to 200 mL.
9. Filter and degas both buffers following the instructions above.

3.4. PolyCAT A Chromatography

1. Equilibrate the column with at least 10 mL polyCAT solution A or until absorbance at 230 nm stabilizes. Use a flow rate of 0.4 mL/min for this step and all steps in the chromatography protocol (see Note 5).
2. While equilibrating, resuspend dried and desalted sample from Subheading 3.2 in 200 μ L polyCAT solution A by vortexing for several seconds (see Note 14).
3. Pellet any particulates in the tube by centrifuging $>15,000 \times g$ for 5 min. Immediately after centrifugation, load the sample into a Hamilton syringe fitted with an appropriate blunt needle point designed for injections into HPLC machines.
4. Inject the sample into the HPLC machine and follow the chromatography protocol outlined in Table 2. Detect the presence of protein by constantly measuring absorbance at 230 nm (see Note 15). Collect fractions every 2 min (0.8 μ L fraction volume) during the first 110 min of the run (see Note 16). Retention time increases

¹²Do not heat the urea solution to dissolve because heating promotes the breakdown of urea into ions that lead to chemical modification (carbamylation) of the proteins being studied.

¹³Deionizing the urea solution is critical to eliminate carbamylation of proteins and to facilitate the interaction of the protein with the column. This resin contains a colorimetric indicator that turns gold or clear when saturated with ions. Continue adding resin until some of the resin remains blue (1–5 g).

¹⁴Samples injected into the column should be free of salts. When possible, we recommend drying the protein sample using a speed-vac centrifuge and resuspending it in polyCAT solution A prior to column injection.

¹⁵When possible, absorbance at 230 nm should be recorded for polyCAT A chromatography of histones, because urea saturates the 214 nm signal.

¹⁶This is not necessary for subsequent runs, but for the initial run we recommend collecting all fractions to help determine the extent of binding and retention in the column.

as the number of acetylated sites on the protein decreases. For example, hyperacetylated proteins will elute before hypoacetylated species.

5. To obtain an optimal range of elution conditions repeat procedures 1–4 above using RP-HPLC-purified protein samples that are not treated with Ac₂O. Compare all fractions from the two polyCAT A chromatography runs by using a dot blot apparatus (load ~10% of each fraction) followed by immunodetection (Fig. 3a). The optimal salt concentration range will encompass fractions that include hyperacetylated (Ac₂O treated) and endogenously acetylated species.
6. Inject more sample into the polyCAT A column and adjust the elution gradient by increasing the time of elution within the newly defined elution range, as described in Fig. 3b (see Note 17). Successful optimization of elution conditions will yield individual peaks on the chromatogram that differ by one acetyl group store fraction at –80°C (Fig. 3b and see Note 18).
7. Desalt each relevant fraction by thawing it individually and injecting it onto an RP-HPLC column using the desalting procedures above followed by drying to completion using a speed-vac centrifuge. Dried fractions may be stored in –80°C.
8. Desalted samples may be analyzed by gel electrophoresis or mass spectrometry (Fig. 3c and ref. 6).

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¹⁷While we narrowed the range of salt concentrations to optimize elution conditions, the samples were still resuspended in polyCAT A “solution A” containing no salt. The details of the optimized polyCAT A chromatography protocol are highlighted in the caption for Fig. 3b. In general, samples should be injected onto the column using a solution that lacks salt. The presence of salt will disrupt the retention time and resolution of the analyte.

¹⁸Following polyCAT A chromatography, we highly recommend washing the column with filtered water (>20 mL) followed by a total system clean of the HPLC, especially if the same HPLC machine will be used for subsequent desalting steps by RP-HPLC. Mixing the mobile phases from each type of chromatography may cause precipitation of salts and significant damage to the column or HPLC pump.

Fig. 1.

In vitro acetylation of proteins using acetic anhydride. **(a)** Unmodified, recombinant histone H3 was treated with acetic anhydride (Ac₂O) and analyzed by dot blot immunodetection using antibodies specific to acetylated histone H3 or total H3. **(b)** The extent of in vitro acetylation of *Tetrahymena* histone H3 was measured by acid-urea gels, which combine size- and charge-based protein separation. The untreated H3 sample contains six bands with each band containing the indicated number of acetyl marks. Treatment with acetic anhydride generates hyperacetylated H3, which is represented by a significant upward shift in acid-urea gels.

Fig. 2. Flowchart of mobile-phase preparation for PolyCAT A chromatography. Circulating *arrows* indicate that stirring is required at that step.

Fig. 3.

Separation of acetylated proteins using polyCAT A chromatography. (a) Dot blot analysis comparing *Tetrahymena* H3 species that were either hyperacetylated in vitro using Ac₂O or endogenously acetylated. These samples represent the extremes of acetylation that may exist in a biologically relevant sample and serve as ideal starting material to optimize polyCAT A chromatography conditions. (b) Chromatogram of an optimized polyCAT A run using 75 µg of endogenously acetylated *Tetrahymena* histone H3. Solutions described in Fig. 2 were used with the following chromatography protocol at a rate of 0.4 mL/min: inject sample; 0% B for 10 min; 0–10% B for 10 min; 10% B for 15 min; 10–50% B for 65 min; 50–100% B for 10 min; 100% B for 10 min; 100–0% B for 5 min; and 0% B for 10 min. Sub-peaks within each major acetylation peak likely represent differently methylated H3 species. (c) Peaks 0–4 from (b) were desalted and analyzed by acid-urea electrophoresis and Coomassie staining.

Table 1

Protocol for desalting by RP-HPLC

Gradient	%B		Volume (mL)	Time (min)
Isocratic	0		4	5
Linear	0	100	24	30
Isocratic	100		4	
Linear	100	0	1.6	2
Isocratic	0		4	5

Use a flow rate of 0.8 mL/min for all steps

Table 2

Protocol for polyCAT A chromatography

Gradient	%B		Volume (mL)	Time (min)
Isocratic	0		4	10
Linear	0	100	36	90
Isocratic	100		4	10
Linear	100	0	0.8	2
Isocratic	0		4	10

Use a flow rate of 0.4 mL/min for all steps