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Separation and characterisation of bovine histone H1 subtypes by combined ion-exchange and reversed-phase chromatography and mass spectrometry

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Abstract

In order to separate and identify histone H1 subtypes from calf thymus we used both electrospray mass spectrometry (ES-MS) and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) after a three-step chromatographic procedure consisting of reversed-phase high-performance liquid chromatography (RP-HPLC), size-exclusion chromatography (SEC) and ion-exchange chromatography (IEC). Under the RP-HPLC conditions described, we obtained two baseline-separated H1-fractions which were characterised by MALDI-TOF-MS. The determined masses ranged from 22 850 to 22 590 for the first fraction and from 22 070 to 21 250 for the second fraction. Further, it was shown that the first fraction contained at least four and the second one at least five subtypes of the histone class H1. Four homogeneous pure H1 subtypes were obtained by a combination of IEC followed by SEC and RP-HPLC. The molecular masses of these four subtypes determined by ES-MS were 22 606, 22 761, 21 347 and 21 263. We obtained six additional molecular masses of histone H1 subtypes from three heterogeneous fractions, namely 22 066, 21 802, 20 586 and 19 817 by ES-MS and 22 800 and 22 675 by MALDI-TOF-MS. The retention times of these fractions and the molecular masses were in agreement with the data obtained from RP-HPLC fractions by MALDI-TOF-MS.

1. Introduction

In recent years the static view of histones as merely structural elements of the chromatin has gradually changed towards a more dynamic role in gene activation and expression [1,2].

Moreover novel extranuclear functions of histones have become apparent; e.g., histone H1 seems to be an integral component of the cytoskeleton as described for sea urchin eggs [3]. The discovery of N-terminal histone sequences in a lectin isolated from human placenta suggests that histones belong to a larger protein family with great functional diversity [4].

The H1 histones are outstanding in view of a high degree of polymorphism already at the gene level resulting in five to eight subtypes in the mammalian species analysed so far [5]. The biological function of the individual subtypes is not well understood, therefore their identifica-

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tion is a matter of great theoretical and practical interest.

Recently, Parseghian et al. [6] presented a classification of H1 subtypes from mammals based on the available protein and DNA sequences and proposed a unifying nomenclature. As to the human histone H1, four subtype sequences were obtained by gene sequencing and four by protein sequencing. Three of these subtypes were obtained at both the gene and the protein level [7–9]. One protein sequence has not yet been found in the genomic material sequenced so far. It is obvious that sequence determinations at both the protein and the gene level will be needed for elucidation the biological function of H1 subtypes; e.g., the differential distribution of H1 subtypes among tissues during different stages of development has to be investigated by means of protein isolation and identification [10]. Further, post-translational modifications, which are numerous in H1 histones, can obviously be identified at the protein level only.

For the bovine H1 subtypes identified so far, their classification is preliminary, since no complete sequences have been obtained, neither at the protein nor at the gene level [6]. Numerous attempts have been reported to isolate and identify bovine H1 subtypes by chromatographic and/or electrophoretic techniques [11–14]. So far, no unambiguous assignment of homogeneous subtypes to chromatographic fractions or electrophoretic bands has been achieved in terms of amino acid sequence or molecular mass.

We now present a three-step chromatographic procedure combined with MS which enabled us to assess the homogeneity of the fractions obtained and to determine the molecular masses of the solutes.

2. Experimental

Sodium chloride p.a. and urea p.a. were purchased from Fluka (Neu Ulm, Germany), disodium hydrogenphosphate–sodium dihydrogenphosphate (both p.a. grade) were purchased from Merck (Darmstadt, Germany). Acetonitrile (grade S) was obtained from Rathburn (Walkerburn, UK), and trifluoroacetic acid (TFA) was purchased from Fluka. Trichloroacetic acid (TCA) and perchloric acid (PCA) (both p.a. grade) were from Merck. Double-distilled water was used throughout.

2.1. Preparation

Calf thymus histone H1 was prepared according to Pehrson and Cole [15] with slight modifications as follows. Calf thymus was homogenized with water (Moulinex, Cologne, Germany). The homogenate was extracted with 7.5% (w/v) PCA by shaking with glass beads at 4°C during 2 h and then centrifuged at 7500 *g* for 10 min (Suprafuge 22, Heraeus Sephatech, Osterode, Germany). The H1 histones were precipitated by adding TCA to a 30% (w/v) final concentration on an ice bath. The precipitate was dialyzed against 1% (v/v) acetic acid and water, freeze-dried and stored at −25°C.

2.2. Electrophoretic analysis of H1 fractions

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) we applied a vertical Midget gel electrophoresis chamber (Pharmacia, Uppsala, Sweden).

The conditions for the SDS-PAGE gels according to Laemmli [16] were $T = 18\%$ and $C = 3\%$ for the separation gels (80 mm × 50 mm × 0.75 mm) and $T = 3.8\%$ and $C = 3\%$ for the stacking gels (80 mm × 15 mm × 0.75 mm). The gels were fixed with 20% TCA (w/v) for 10 min, stained with Coomassie Blue for at least 1 h and destained with acetic acid–ethanol–water (1:3:6, v/v/v).

2.3. RP-HPLC of histones

All RP-HPLC experiments were performed on a Waters HPLC system consisting of a 600 multisolvent delivery system, a Lambda-Max 481 LC spectrometer and a 740 integrator data module (Waters, Eschborn, Germany). For analytical RP-HPLC a Nucleosil column (260 × 4 mm I.D., pore size 10 nm, octadecyl, particle size 5 μm) (Knauer, Berlin, Germany) and for

semi-preparative RP-HPLC an Eurosil Bioselect column (150 × 20 mm I.D., pore size 20 nm, octadecyl, particle size 10 μm) (Eurochrom, Berlin, Germany) was used. The chromatography was performed at 25°C with the following flow-rates, wavelengths and gradients: Nucleosil column, 1 ml/min, 220 nm, 170 MPa, 8 min linear from 20 to 32% acetonitrile (0.1% TFA), followed by 28 min linear from 32 to 37.6% acetonitrile (0.1% TFA); Eurosil column, 6 ml/min, 227 nm, 225 MPa, 40 min linear from 20 to 60% acetonitrile (0.1% TFA).

2.4. Ion-exchange and size-exclusion chromatography of H1 histones

For IEC and SEC a FPLC system (Pharmacia) was used consisting of a LCC 500 controller, two P-500 pumps, an Uvicord SII detector (226 nm) and a LKB 2221 integrator. The Superose 12HR30 gel filtration column was also obtained from Pharmacia.

For the semi-preparative strong cation exchanger we used a self-packed 70-ml column with Macro Prep 50 S material (13 cm × 26 mm I.D., pore size 100 nm, particle size 50 μm, BioRad, Munich, Germany). The fractions were eluted at a flow-rate of 3.5 ml/min starting from eluent A (0.1 mol l^{-1} sodium phosphate buffer pH 7.0, 1 mol l^{-1} urea) to eluent B (0.1 mol l^{-1} sodium phosphate buffer pH 7.0, 1 mol l^{-1} urea, 1 mol l^{-1} sodium chloride). With the shallower gradient mentioned in the Results section the sodium chloride concentration of eluent A was 0.5 mol l^{-1} and that of B was 0.8 mol l^{-1}. The gradient was 0.5 mol l^{-1} to 0.8 mol l^{-1} in 162 min.

After IEC all fractions were desalted on Superose 12HR30 (30 cm × 12 mm I.D., 0.05% TFA, 0.6 ml/min, 226 nm). The freeze-dried fractions were dissolved in 450 μl of 0.1% TFA and further separated by RP-HPLC with the Eurosil column as described above.

2.5. Mass spectrometry

The electrospray mass spectra were obtained using a TSQ 700 spectrometer coupled to an electrospray interface (Finnigan MAT, Bremen, Germany). The needle voltage was 4.5 kV, and the capillary temperature was kept at 200°C. The proteins were dissolved in a 1:1 mixture of methanol–acetic acid to a concentration of 10 pmol/μl and injected at a flow-rate of 5 μl/min by a Harvard syringe pump. Sheath gas pressure was adjusted to 8.6 MPa, no sheath liquid was added. The spectra were averaged for 1 min, before written to disk.

MALDI was coupled with TOF for mass analysis on a Vision 2000 spectrometer (Finnigan MAT). A N_2-LST laser (337 nm) and 2,5-dihydroxybenzoic acid (DHB) as matrix were used.

3. Results

3.1. Reversed-phase chromatography

The reversed-phase separation yielded a baseline separation of two H1 fractions which

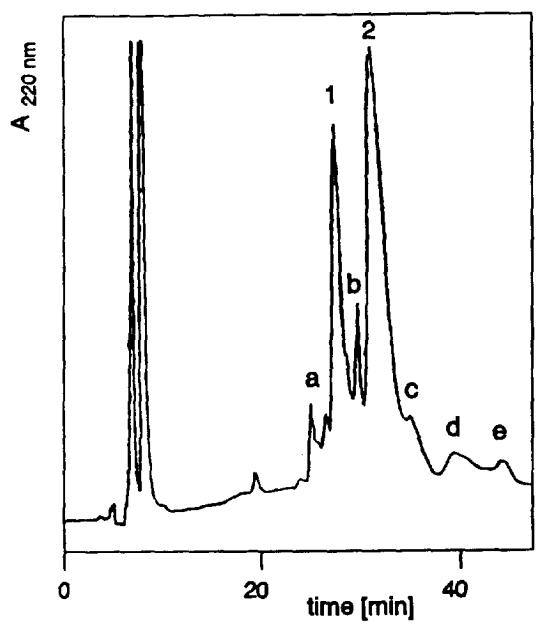

Fig. 1. Fractionation of crude H1 histones by RP-HPLC. A 0.2-mg sample was dissolved in 450 μl of 0.1% TFA and fractionated on the Nucleosil column (for conditions see Experimental). The numbers 1 and 2 for the two histone H1 fractions have also been used for the RP-HPLC fractions obtained by the three-step chromatographic procedure (IEC–SEC–RP-HPLC) with the semi-preparative Eurosil column.

eluted behind the HMG proteins and before the core histones identified by SDS-PAGE (Fig. 1). Whereas the HMG proteins were baseline-separated the core histones were not well separated (Fig. 2). Shallower gradients did not affect the resolution. In some acid extracts we observed a further fraction between the two H1 fractions identified as ubiquitin by ion-spray MS, SDS-PAGE and N-terminal protein sequence analysis. All other RP-HPLC fractions had the same retention times for material from different preparations. The upscaling to the semi-preparative Eurosil column resulted in a reproducible fractionation comparable with that of the Nucleosil column. The acetonitrile percentage necessary for eluting the sample was higher on the Eurosil-column.

The H1 fractions from RP-HPLC had the following pattern in SDS-PAGE: the first fraction resulted in the upper band and the second fraction in a double band, typical for histone H1 from calf thymus and most other species. Dividing the second peak in two fractions, namely the ascending and the descending part, the ascending part yielded the upper and the descending part the lower band in SDS-PAGE (Fig. 3).

Because of the limitation of chromatographic and electrophoretic techniques to characterise the subtypes of H1 we used MALDI-TOF-MS to further characterise the various fractions obtained. Fig. 4 shows that the first fraction of the RP-HPLC separation represented a protein mixture in the mass range of 22 850 to 22 590 with no other impurities. Within this range the masses of 22 800, 22 675 and 22 595 could be clearly identified. Similarly, the second RP-HPLC fraction showed no impurities and a mass distribution between 22 070 and 21 250 with identified species of 22 051, 21 790 and 21 252 (Fig. 5). However, each of the three peaks in Fig. 5 is inhomogeneous indicating the presence of at least three additional subtypes. Thus, at least nine subtypes had been present in the preparation.

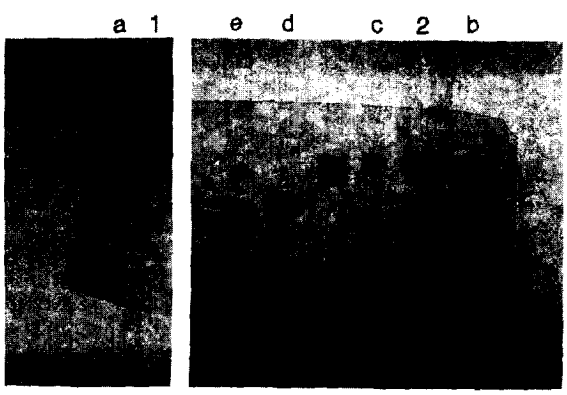

Fig. 2. SDS-PAGE of the protein fractions of Fig. 1. Each designated fraction was electrophoresed on a 18% polyacrylamide gel as described under Experimental. The lanes are designated according to the fractions.

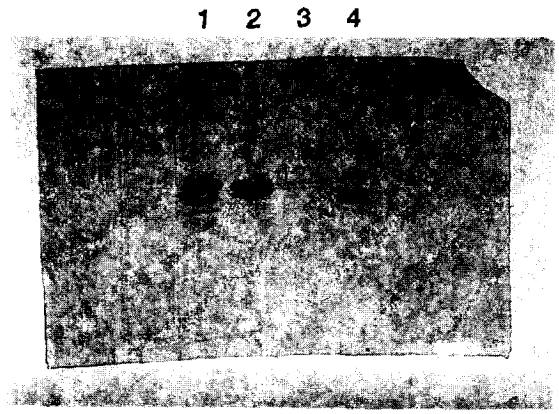

Fig. 3. SDS-PAGE on a 18% polyacrylamide gel of fractions 1 and 2 of Fig. 1. Lane 1, fraction 1; lane 2, fraction 2; lane 3, fraction 2 ascending part; lane 4, fraction 2 descending part.

3.2. Ion-exchange chromatography

The material obtained by the preparation was separated into four fractions (Fig. 6). The histones H1 eluted in fraction 4 were well separated from the accompanying substances with no further separation at all. From the single H1 peak two fractions were taken i.e., the ascending part designated A and the descending part designated B and were further separated by RP-HPLC. The ascending part yielded two and the descending

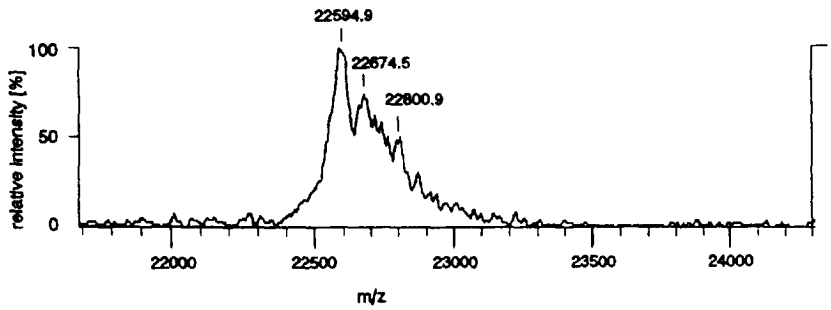

Fig. 4. MALDI-TOF-MS spectrum of fraction 1 of Fig. 1 with a molecular mass range from 22 850 to 22 590. 2,5-Dihydroxybenzoic acid was used as matrix, the laser wavelength was 337 nm.

part three fractions, designated A1, A2, B1, B2 and B3, respectively. Of these five fractions two were characterised by ES-MS (Table 1). The ES-MS analysis of fraction A1 yielded a single molecular mass of 22 606 (Fig. 7). Fraction B2 yielded the molecular masses of 22 066, 21 802 and 21 267 showing that this fraction contains more than one subtype (Fig. 8).

Because of this obvious complexity in a further experiment the H1 eluate of cation-exchange chromatography was collected in six consecutive fractions designated C, D, E, F, G and H by a shallower gradient, as described in Experimental. Each of the six fractions (C–H) yielded two fractions by RP-HPLC, designated C1, C2, . . . , H2 which were examined by ES-MS. The identified molecular species and their masses are given in Table 1.

The range of all molecular masses obtained via the combined IEC–SEC–RP-HPLC procedure corresponded with the results obtained via RP-HPLC alone. The molecular masses of the earlier eluting RP-HPLC fractions (C1–H1) were in the range 22 850–22 590 and the molecular masses of the next RP-HPLC fractions (C2–H2) were in the range of 22 070–21 250. The two subtypes marked with brackets (fraction F2) are outside the mass range obtained by MALDI-TOF-MS.

Altogether we obtained ten molecular species with molecular differences exceeding 39 Da (~0.2%) of the following molecular masses: 22 800, 22 761, 22 675, 22 606, 22 066, 21 802, 21 347, 21 263, 20 586 and 19 817. From all RP-HPLC fractions obtained, nine were homogeneous in the MS experiment, of which six had the same molecular mass of 22 606. The distribution of the subtypes is in agreement with the

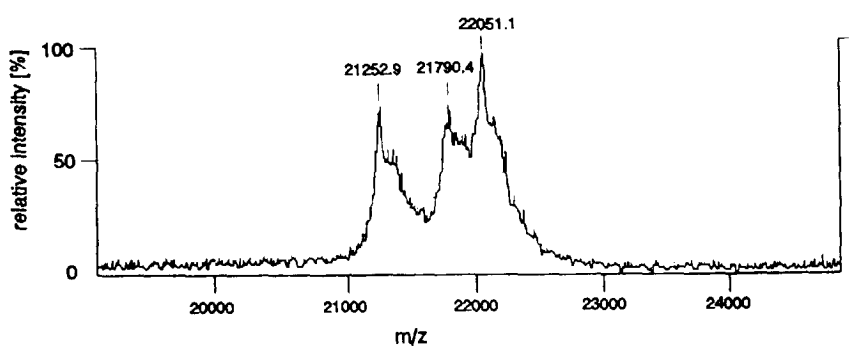

Fig. 5. MALDI-TOF-MS spectrum of fraction 2 of Fig. 1 with a molecular mass range from 22 070 to 21 250. 2,5-Dihydroxybenzoic acid was used as matrix, the laser wavelength was 337 nm.

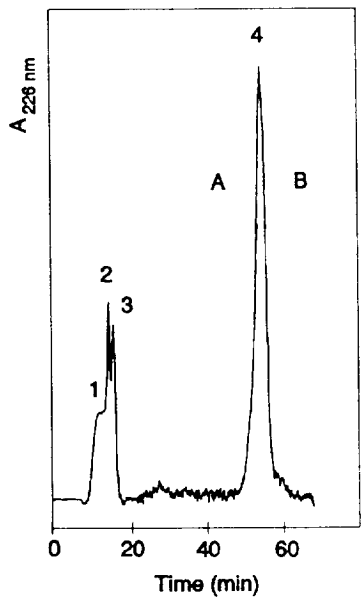

Fig. 6. IEC of histone H1 prepared according to Pehrson and Cole [15]. A 7-mg amount of the crude H1 histones was dissolved in 5 ml of eluent A before injection. The gradient was 0 to 1 mol l^{-1} NaCl in 60 min in the buffers as described in Experimental. The ascending fraction was designated A and the descending fraction was designated B.

Table 1
Molecular masses of bovine histone H1 subtypes obtained by mass spectrometry

Fraction No.	Mass	MS technique	Fig. No.
1	22 850–22 590 22 800, 22 675, 22 595	MALDI-TOF	4
2	22 070–21 250 22 051, 21 790, 21 252	MALDI-TOF	5
A1	22 606	ES	7
A2	Not detected		
B1, B3	Not detected		
B2	22 066, 21 802, 21 267	ES	8
C1	22 761	ES	
C2	21 347	ES	
D1	22 604	ES	
D2	21 263	ES	
E1	22 606	ES	
E2	21 269, 22 073	ES	
F1	22 607	ES	
F2	[19 817, 20 586]	ES	
G1	22 606	ES	
G2	Not detected	ES	
H1	22 605	ES	
H2	21 802, 22 066	ES	

The letters A–H designate the fractions from ion-exchange chromatography and the numbers 1 and 2 those from RP-HPLC. (MALDI-TOF: matrix-assisted laser desorption ionisation time-of-flight, ES: electrospray).

results of MALDI-TOF-MS whereas more than three subtypes have masses above 22 590 and at least six have masses below 22 070.

4. Discussion

In accordance with other authors [17–20] we found that homogeneous fractions obtained by RP-HPLC or IEC of histone H1 appeared homogeneous in SDS-PAGE. Hitherto, this has commonly been taken as proof for molecular homogeneity and led to the assignment of H1 subtypes with respect to chromatographic fractions or bands obtained by SDS-PAGE. Our data show convincingly that this seems generally not feasible. Comparison of the molecular masses from both methods reveals a systematic difference of about 15 mass units which is still within the accepted error limit of MALDI-TOF-MS.

At the present state of knowledge it is not possible to make assignments of the obtained molecular masses with respect to bovine H1 subtypes, since neither complete amino acid sequences nor gene sequences are known for any of these subtypes. Therefore, the fractions obtained by the different chromatographic techniques may also contain chemically modified molecular species, e.g., ES-MS analysis of murine histone H1 fractions led Giancotti et al. [10] to identify subtypes with up to five phosphate residues. These are the first determined molecular masses of bovine histone H1 subtypes. Our separation scheme permits the isolation of sufficient amounts of at least four homogeneous protein fractions from bovine H1 as prerequisite for amino acid sequence determination which is underway in our laboratory.

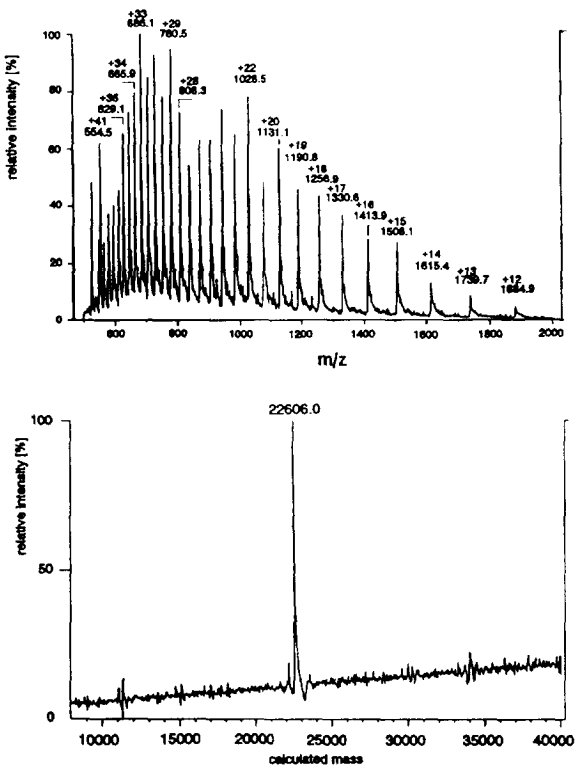

Fig. 7. ES-MS of A1. From the 29 multiprotonated ions carrying 12$^+$ (m/z 1184) to 41$^+$ (m/z 554.5), a molecular mass of 22 606.0 ± 1.1 was calculated.

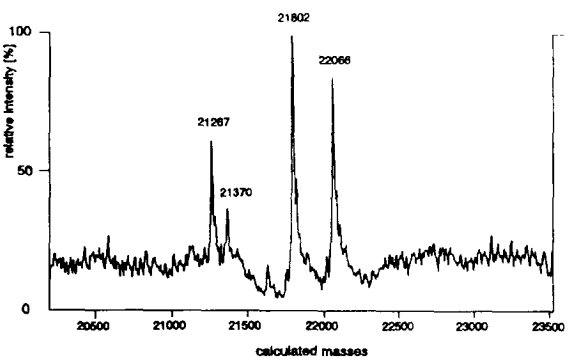

Fig. 8. ES-MS of B2. From this protein mixture three molecular masses were calculated: 22 066, 21 802 and 21 267.

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