ELSEVIER

Journal of Chromatography A, 743 (1996) 137–144

JOURNAL OF CHROMATOGRAPHY A

Separation of acetylated core histones by hydrophilic-interaction liquid chromatography

Herbert Lindner*, Bettina Sarg, Christoph Meraner, Wilfried Helliger

Institute of Medical Chemistry and Biochemistry, Fritz Preglstrasse 3, A-6020 Innsbruck, Austria

Abstract

Hydrophilic-interaction liquid chromatography (HILIC) has recently been introduced as a highly efficient chromatographic technique for the separation of a wide range of solutes. The present work was performed with the aim of evaluating the potential utility of HILIC for the separation of posttranslationally acetylated histones. The protein fractionations were generally achieved by using a weak cation-exchange column and an increasing sodium perchlorate gradient system in the presence of acetonitrile (70%, v/v) at pH 3.0. In combination with reversed-phase high-performance liquid chromatography (RP-HPLC) we have successfully separated various H2A variants and posttranslationally acetylated forms of H2A variants and H4 proteins in very pure form. An unambiguous assignment of the histone fractions obtained was performed using high-performance capillary and acid-urea–Triton gel electrophoresis. Our results demonstrate that for the analysis and isolation of modified core histone variants HILIC provides a new and important alternative to traditional separation techniques and will be useful in studying the biological function of histone acetylation.

Keywords: Hydrophilic-interaction liquid chromatography; Histones; Protein modifications; Acetylation; Ion-exchange chromatography

1. Introduction

Histones are the most intensively studied group of basic nuclear proteins and are of great importance with regard to the organization of chromatin structure and control of gene activity (for a review, see [1]). Extensive chromatographic and electrophoretic studies have demonstrated that they can be resolved into five main classes: H1, H2A, H2B, H3 and H4. In mammals, each class of these nuclear proteins, with the exception of H4, is represented by non-allelic primary sequence variants or subtypes which differ one from the other only slightly in molecular size and primary structure. Moreover, all histone proteins are known to be posttranslationally modified, such as acetylated, phosphorylated, ADP-ribosylated etc. These modifications alter the amino acid side chains, thereby creating histones whose functional properties may differ. One of the major types of modification observed is reversible acetylation of certain lysine residues within the N-terminal domains of the core histones (H2A, H2B, H3, H4). This modification has been associated with a transcriptionally active state of chromatin [2–6]. In addition to transcription, histone acetylation has been implicated in other cellular processes such as replication and histone replacement during differentiation [7,8].

So far, the biological function of histone acetylation is not well understood. To study nucleosome and chromatin structure–function relationships of histone acetylation it is necessary to assemble nu-

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00131-8

cleosomes and chromatin models from exactly defined acetylated histones and DNA sequences. This requires the development of chromatographic methods for the fractionation and isolation of the various acetylated core histone forms. During the last three decades many attempts have been made to separate all histone subtypes and their modified derivatives. Until recently, the separation techniques were limited to gel electrophoresis and conventional ion-exchange chromatography. Gel electrophoresis permits separation of several acetylated and phosphorylated forms of histones with the drawbacks generally known. The low-pressure ion-exchange chromatography of histones is often unsatisfactory and moreover, extremely time consuming [9,10]. HPLC isolation of acetylated H3 and H4 proteins was performed by Marvin et al. [11]. A satisfying resolution, however, was only brought about by adding 8 *M* urea.

A new method, hydrophilic-interaction liquid chromatography (HILIC), combining hydrophilic stationary phases and hydrophobic, mostly organic mobile phases, was introduced by Alpert [12] and opened new prospects on the separation of biomolecules. Separation by HILIC, in a manner similar to normal-phase chromatography, depends on hydrophilic interactions between the solutes and the hydrophilic stationary phase. When using an ion-exchange column, not only HILIC but also electrostatic effects come into play. Such mixed-mode chromatography allows the efficient resolution of various biomolecules [13,14]. In this paper we demonstrate that the HILIC system represents a fast and efficient method for the separation and isolation of H2A subtypes and the acetylated states of histones H2A and H4.

2. Materials and methods

2.1. Chemicals

Sodium perchlorate ($NaClO_4$) and triethylamine (TEA) were purchased from Fluka (Buchs, Switzerland), hydroxypropylmethyl cellulose (HPMC; 4000 cP) and trifluoroacetic acid (TFA) were obtained from Sigma (Munich, Germany) and ethylene glycol monomethyl ether (EGME) was ordered from Aldrich (Steinheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Cell line and culture conditions

Friend erythroleukaemic cells (line B8) were grown in Dulbecco's MEM containing 2×non-essential amino acids, 1×penicillin–streptomycin and 10% FCS. Cells were cultured with initial cell densities of $5 \cdot 10^4$–$7 \cdot 10^4$ cells per ml at 37°C and 5% CO_2. In order to achieve hyper-acetylated core histones 1.75 m*M* sodium butyrate was added for 24 h to cells growing exponentially at a density of $2 \cdot 10^5$ cells per ml.

2.3. Sample preparation

Whole histones from Friend erythroleukaemic cells were prepared as described previously [15].

2.4. High-performance liquid chromatography

The equipment used consisted of two 114M pumps, a 421A system controller and a Model 165 variable-wavelength UV-Vis detector (Beckman Instruments, Palo Alto, CA, USA). The effluent was monitored at 210 nm and the peaks were recorded using a Beckman System Gold software.

2.5. Reversed-phase HPLC

The separation of whole histones from untreated logarithmically growing and from butyrate-treated Friend erythroleukaemic cells was performed on a Nucleosil 300-5 C_4 column (125×8 mm I.D., 5 μm beads, 30 nm; Macherey-Nagel, Düren, Germany). The lyophilized proteins were dissolved in water containing 0.2 *M* 2-mercaptoethanol, and samples of 250 μg were injected onto the column. The histone sample was chromatographed within 100 min at a constant flow of 1.0 ml/min with a multi-step acetonitrile gradient starting at 40% solvent B, (solvent A: water containing 0.1% (v/v) TFA and 10% (v/v) EGME; solvent B: 70% (v/v) acetonitrile, 0.1% (v/v) TFA and 10% (v/v) EGME). The concentration of solvent B was increased linearly in the following order: from 40 to 61% (45 min), from 61 to 64% (35 min), from 64 to 74% (2 min),

maintained at 74% for 9 min and from 74 to 100% (10 min). Histone fractions were collected and after the addition of 25 μl 2-mercaptoethanol (0.2 *M*), lyophilized and stored at −20°C.

2.6. Hydrophilic-interaction liquid chromatography

Histone fractions H4, H2A.1 and H2A.2, isolated by RP-HPLC, were analysed on a SynChropak CM300 column (250×4.6 mm, 6.5 μm beads, 30 nm; Hewlett-Packard, Vienna, Austria) at a constant flow-rate of 1.0 ml/min.

H4 fractions were eluted within 35 min using a two-step gradient starting from 0% B (solvent A: 70% acetonitrile, 0.015 *M* triethylamine phosphate (TEAP, pH 3.0); solvent B: 70% acetonitrile, 0.015 *M* TEAP (pH 3.0) and 0.68 *M* $NaClO_4$). The concentration of solvent B was increased from 0 to 10% during 2 min and from 10 to 40% during 30 min.

The H2A variants were separated at 18°C with the solvents described above. H2A.2 was analysed using a two-step gradient from 0 to 35% B (5 min) and from 35 to 48% B (25 min). H2A.1 was separated within 30 min running a two-step gradient from 0 to 30% B (5 min) and from 30 to 45% B (25 min). The isolated protein fractions were desalted using RP-HPLC.

Histone fractions obtained in this way were collected and after the addition of 25 μl 2-mercaptoethanol (0.2 *M*), lyophilized and stored at −20°C.

2.7. Capillary electrophoresis

High-performance capillary electrophoresis (HPCE) was performed on a Beckman system P/ACE 2100 controlled by an AT486 computer. Data collection and post-run data analysis were carried out using P/ACE and System Gold software (Beckman Instruments). The capillary cartridge used was fitted with 75 μm I.D. fused-silica of 57 cm total length (50 cm to the detector). Protein samples were injected by pressure and on-column detection was performed by measuring UV absorption at 200 nm. An untreated capillary was used in all experiments. Runs were carried out in 100 m*M* phosphate buffer (pH 2.0) containing 0.02% HPMC at constant voltage (12 kV) and at a capillary temperature of 25°C.

3. Results and discussion

Up to now, no chromatographic technique permits the simultaneous separation of all histones and their modified forms. RP-HPLC has already proved useful for the separation of core and linker histones as well as for the resolution of certain histone variants [16–20]. This procedure, however, does not allow resolution of posttranslationally modified histones. To achieve this objective, in a first step we applied RP-HPLC in order to fractionate whole histones isolated from erythroleukaemic cells. Using a semi-preparative column filled with Nucleosil 300-5 C_4 and a multi-step water–acetonitrile gradient we obtained eleven histone fractions, namely the linker histone subtypes H1°, H1b, H1a, H1e+H1d, H1c and the core histones H2B, H2A.2, H4, H2A.1, H3.2+H3.3 and H3.1 (Fig. 1) which were characterized by sodium dodecylsulfate (SDS) and AUT polyacrylamide gel electrophoresis, as recently described [19]. The fact that variously acetylated forms of one and the same protein are coeluted in RP-HPLC with its unmodified parent protein has previously been demonstrated for histone H4. By applying a new HPCE method (uncoated capillary, 0.1 *M* phosphate buffer with pH 2.0 containing 0.02% HPMC), the

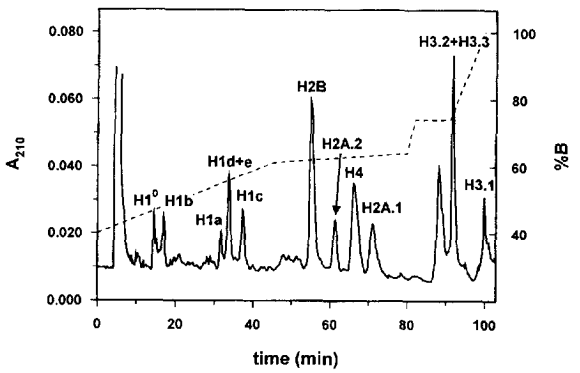

Fig. 1. Separation of whole histones by RP-HPLC. Amounts of 250 μg of histone sample isolated from untreated Friend erythroleukaemic cells were injected on to a Nucleosil 300-5 C_4 column. Flow-rate, 1 ml/min. Monitoring wavelength: 210 nm. Eluent: 100 min multi-step gradient, where solvent A is water and solvent B is 70% acetonitrile, both solvents containing 0.1% TFA and 10% EGME. Starting at 60% A–40% B, the concentration of solvent B was increased linearly in the following order: from 40 to 61% (45 min), from 61 to 64% (35 min), from 64 to 74% (2 min), maintained at 74% for 9 min and from 74 to 100% (10 min).

histone H4 fraction, which was a single peak in RP-HPLC, was further separated into three peaks belonging to the non-, mono- and di-acetylated forms of histone H4 [21].

In a first effort the H4 sample was chromatographed under ion-exchange conditions applying several silica-based cation-exchange columns with a linear 0-2 *M* sodium chloride gradient. These attempts, however, were without success due to irreversible adsorption of the histone (data not shown). We assume that this effect is caused by strong hydrophobic interactions between column matrix and the C-terminal region of histone H4 which is enriched in acidic and hydrophobic amino acids.

Since HILIC-based methods have recently been reported for the separation of amino acids, peptides and carbohydrates [12], we were interested in testing the HILIC mode for the separation of acetylated histones for two reasons. First, in contrast to ion-exchange chromatography the HILIC system separates solutes based on their hydrophilicity. In the case of histone acetylation not only the net positive charge but also the hydrophilicity of histone proteins will be changed in the same manner, the higher the degree of acetylation, the lower the positive charge and – more important in this context – the hydrophilicity of the histone molecule. Second, all histones display multi-domain structures, an extended hydrophobic domain is flanked by one or two hydrophilic basic regions. These regions should preferentially bind to the hydrophilic chromatographic sorbent. Since acetylation occurs exclusively in the hydrophilic N-terminal region of histones, thus changing the hydrophilicity of their contact region, the HILIC mode should be able to discriminate between individual modified proteins. Furthermore, this concept could explain the poor ability of RP-HPLC to resolve histone modifications. In this case, the hydrophobic domain which is not affected by acetylation, accounts for adsorption to the hydrophobic stationary phase. In fact, when sodium perchlorate was used instead of chloride in the presence of at least 50% acetonitrile, which are already typical HILIC conditions, resolution improved. Interestingly, a remarkable difference in the elution behavior of histone H4 was observed, depending on the column type used. Best results were obtained with the SynChropak CM300 column, a weak silica-based cation-exchange column having carboxymethyl functionalities. When other column types were used, however, the H4 protein was eluted markedly later and accompanied by a dramatically reduced protein recovery (data not shown). This inconvenient effect can easily be explained by a precipitation of proteins caused by the higher perchlorate concentration required for histone elution. Under optimized conditions applying the SynChropak CM300 column with a linear sodium perchlorate gradient (0–0.68 *M*) in the presence of 70% acetonitrile and 0.015 *M* TEAP (pH 3.0), the H4 fraction was separated into two major and some minor peaks (Fig. 2). Assignment of the individual fractions obtained by HILIC was performed in a 3-fold way. First, after desalting by RP-HPLC the isolated HILIC fractions were subjected to CE analysis and their migration times compared with those of the whole histone H4 sample. As an example, CE analysis of the second highest peak in Fig. 2 (eluted at 26 min and designated peak 2) is shown in Fig. 3a. The fraction has been proved to be remarkably pure, only a single peak was obtained, having a migration time nearly identical to that of the mono-acetylated histone H4 (Fig. 3b). Second, assignment was confirmed by adding individual HILIC fractions to the whole H4 histone sample leading to a characteristic increase of one peak in the electropherogram (shown in Fig. 3c for the unknown peak 2 in Fig. 2). Third, the same HILIC fractions

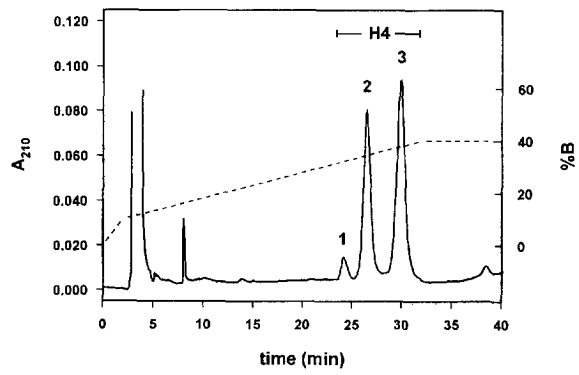

Fig. 2. Separation of acetylated histone H4 by HILIC. The histone H4 fraction used was isolated by RP-HPLC (Fig. 1). Column, SynChropak CM300 (250×4.6 mm, 6.5 μm beads, 30 nm); flow-rate, 1.0 ml/min; temperature, 20°C; mobile phase, 0.015 *M* TEAP (pH 3.0) with 70% (v/v) acetonitrile and a gradient of sodium perchlorate (see Section 2).

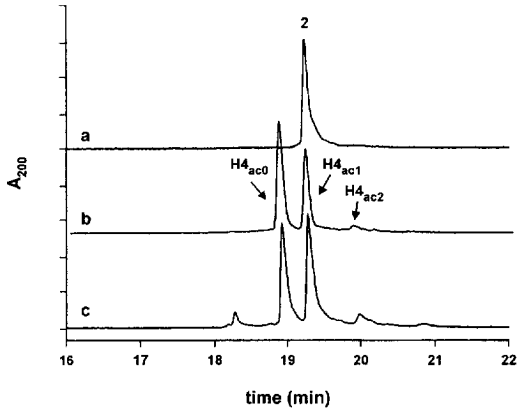

Fig. 3. HPCE of acetylated histone H4. CE conditions were as follows: buffer, 0.1 *M* sodium phosphate (pH 2.0) with 0.02% HPMC; injection time, 3 s; voltage applied, 12 kV; constant temperature, 25°C; detection at 200 nm; untreated capillary (50 cm×75 μm I.D.). (a) HPCE of the H4 fraction eluted at 26 min in the HILIC run of Fig. 2; (b) HPCE of histone H4 isolated by RP-HPLC (Fig. 1); (c) HPCE of histone H4 isolated by RP-HPLC with the addition of the H4 fraction eluted at 26 min in the HILIC run shown in Fig. 2; $H4_{ac0}$, $H4_{ac1}$, $H4_{ac2}$=non-, mono- and diacetylated H4 histones.

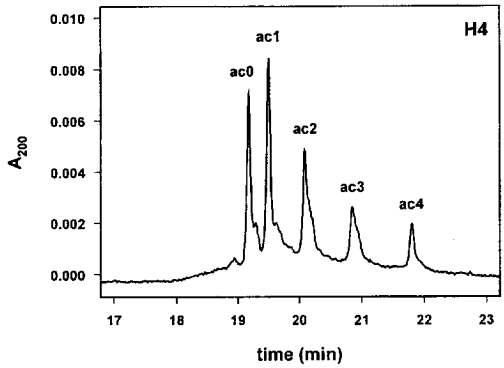

Fig. 4. HPCE of hyper-acetylated histone H4 of butyrate-treated Friend erythroleukaemic cells. The histone H4 fraction used was isolated by RP-HPLC. CE conditions as in Fig. 3; ac0, ac1, ac2, ac3, ac4= non-, mono-, di-, tri- and tetraacetylated H4 histones.

were analysed individually by AUT–PAGE (data not shown). All these procedures led to the following assignment of histone H4 peaks shown in Fig. 2. The first eluted small peak consisted of di-acetylated H4 (peak 1), followed by the mono-acetylated (peak 2) and the non-acetylated form (peak 3).

To examine the analytical potential of the HILIC method developed, we subsequently attempted to separate highly acetylated histone H4 into its distinct forms. To achieve hyper-acetylated core histones, Friend erythroleukaemic cells were treated with sodium butyrate which is known to be a potent inhibitor of histone deacetylase(s) [22]. The H4 fraction was isolated by RP-HPLC and the degree of acetylation determined by CE (Fig. 4). Five peaks were found and identified as the non-, mono-, di-, tri- and tetraacetylated form of histone H4 according to [21]. The corresponding HILIC fractionation of the histone H4 sample resulted in a similar separation pattern. As shown in Fig. 5, five baseline separated peaks were obtained, which were identified as described above with the following result: tetra-acetylated histone H4 (ac_4) was eluted first, followed by the tri-(ac_3), di-(ac_2), mono-(ac_1) and non-acetylated (ac_0) derivatives. These identities are marked on Fig. 5. The AUT–PAGE shown in Fig. 6 illustrates the purity of the histone H4 fractions isolated by the HILIC procedure described here.

It should be pointed out that in CE (Fig. 4) the peak order of hyper-acetylated H4 histones is reversed as compared to that in the HILIC mode (Fig. 5). As described above, this observation can be explained by the fact that the acetylation of lysine residues of histone H4, depending on the degree of acetylation, causes a decrease of both the overall positive charge and the hydrophilicity of the molecule. Whereas proteins are separated in CE in order of decreasing net positive charge, in the HILIC mode

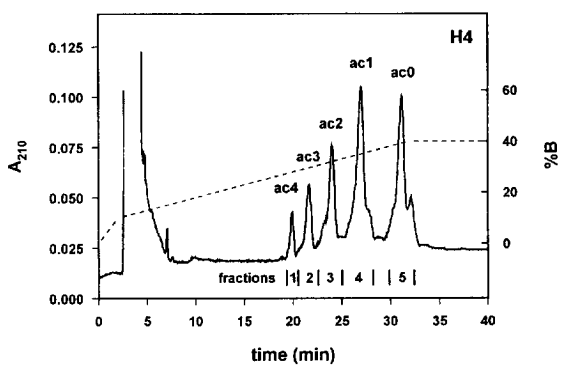

Fig. 5. Separation of hyper-acetylated histone H4 of butyrate-treated Friend erythroleukaemic cells by HILIC. The histone H4 fraction was isolated by RP-HPLC. Conditions were the same as for Fig. 2. Abbreviations used were the same as for Fig. 4. Numbered fractions were lyophilized and subjected to AUT–PAGE (shown in Fig. 6).

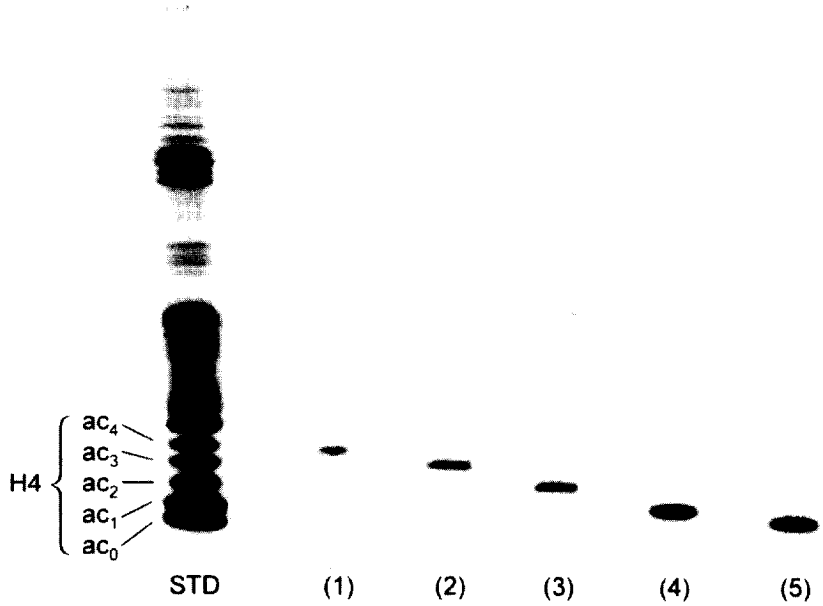

Fig. 6. AUT–PAGE of hyper-acetylated histone H4 separated by the HILIC system used in Fig. 5. (1)–(5), number of effluent fractions, as designated in Fig. 5. Lane STD, whole histones (15 μg) from butyrate-treated Friend erythroleukaemic cells.

proteins are eluted in order of increasing hydrophilicity.

The fact that the HILIC method presented in this paper is not limited to the separation and isolation of modified H4 histones is shown in Fig. 7. The histone H2A.1 fraction obtained by RP-HPLC was analysed using the SynChropak CM300 column and HILIC conditions similar to those applied for the fractionation of hyper-acetylated histone H4 (Fig. 5). As can be seen, we were successful in baseline separation of non-acetylated histone H2A.1 from the mono-acetylated one and the non-acetylated histone H2A.X (=H2A.4) from both mono-acetylated H2A.1 and H2A.X histones. Identification of the individual peaks followed from AUT gel electrophoresis (Fig. 8).

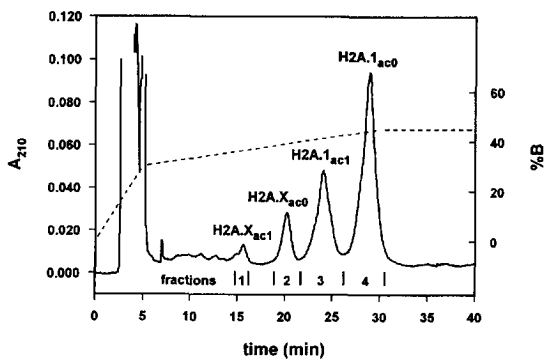

Fig. 7. Separation of histone H2A.1 of butyrate-treated Friend erythroleukaemic cells by HILIC. The histone H2A.1 fraction was isolated by RP-HPLC. Conditions were the same as for Fig. 2 except that the gradient used was modified (see Section 2) and column temperature was 18°C; H2A.1_{ac0}, H2A.1_{ac1}, H2A.X_{ac0}, H2A.X_{ac1}: non- and mono-acetylated histones H2A.1 and H2A.X, respectively. Numbered fractions were lyophilized and subjected to AUT–PAGE (shown in Fig. 8).

Up to now, the chromatographic fractionation of the complex histone H2A.2 variants has been an unresolved problem as well. On very long AUT gels, H2A.2 can be partially resolved into the two subcomponents H2A.2^a and H2A.2^b [23], most likely differing in the presence or deletion of His^{-124} as shown in human H2A [24]. This gel system, however, is unsuitable for separating non-acetylated H2A.2^b from mono-acetylated H2A.2^a. By applying the HILIC method to separation of the H2A.2 fraction isolated by RP-HPLC, a complete resolution of non- and mono-acetylated H2A.2^a and H2A.2^b histones from each other was achieved. Fig. 9 shows the chromatogram. The five peaks obtained within 25 min were identified by AUT gel electrophoresis (Fig.

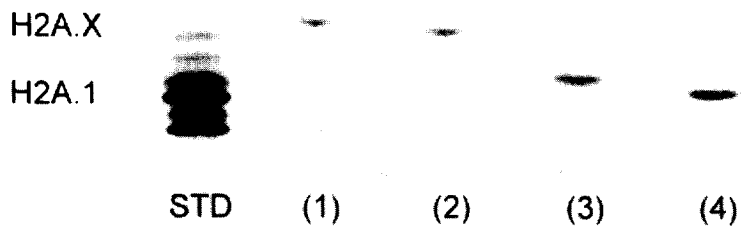

Fig. 8. AUT–PAGE of histone H2A.1 separated by the HILIC system used in Fig. 7. (1–4) Number of effluent fractions, as designated in Fig. 7. Lane STD, H2A histone (8 μg) from butyrate-treated Friend erythroleukaemic cells.

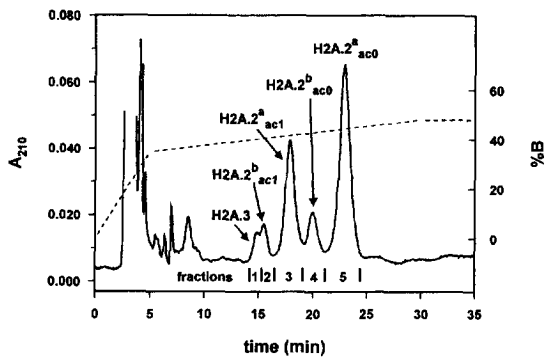

Fig. 9. Separation of histone H2A.2 of butyrate-treated Friend erythroleukaemic cells by HILIC. The histone H2A.2 fraction was isolated by RP-HPLC. Conditions were the same as for Fig. 2 except that the gradient used was modified (see Section 2) and column temperature was 18°C; $H2A.2^{a}_{ac0}$, $H2A.2^{a}_{ac1}$, $H2A.2^{b}_{ac0}$, $H2A.2^{b}_{ac1}$: non- and mono-acetylated histones $H2A.2^{a}$ and $H2A.2^{b}$, respectively. Numbered fractions were lyophilized and subjected to AUT–PAGE (shown in Fig. 10).

10) with the following result: fraction No. 1 most likely contains H2A.3; No. 2, mono-acetylated histone $H2A.2^{b}$, which is not well separated from H2A.3; No. 3, mono-acetylated $H2A.2^{a}$; No. 4, non-acetylated $H2A.2^{b}$; No. 5, non-acetylated $H2A.2^{a}$.

Histone H2A.3 occurs in very low amounts in rapidly dividing cells but accumulates slowly in non-dividing cells [22,24]. To ascertain that fraction 1 consists of histone H2A.3 we isolated an H2A.2 fraction from logarithmically growing cells by RP-HPLC and analysed it by HILIC (data not shown). Since the peak in question decreases obviously, the presence of H2A.3 in fraction 1 appears to be supported.

Finally, the H2A.2 fraction already analysed by HILIC (Fig. 9) was subjected to CE using the same conditions as for separation of acetylated H4 histones. Fig. 11 shows the resulting electropherogram. As expected, the peak order was reversed compared to that in the HILIC mode (Fig. 9). It should be noted, however, that in contrast to HILIC, CE clearly separated histone H2A.3 from mono-acetylated $H2A.2^{b}$.

4. Conclusion

The results presented demonstrate that the HILIC system, in combination with RP-HPLC, is a particularly powerful and reliable technique for the

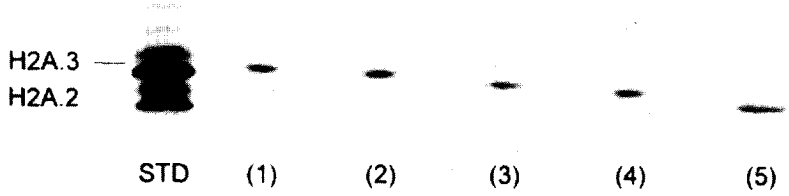

Fig. 10. AUT–PAGE of histone H2A.2 separated by the HILIC system used in Fig. 9. (1–5) Number of effluent fractions, as designated in Fig. 9. Lane STD, H2A histone (8 μg) from butyrate-treated Friend erythroleukaemic cells.

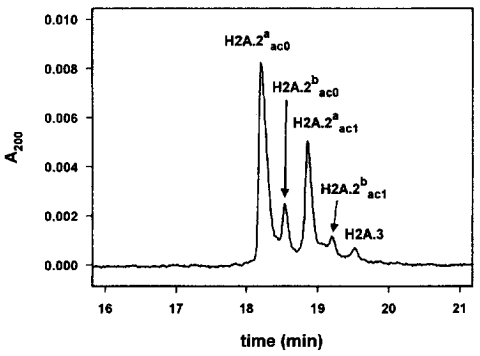

Fig. 11. HPCE of histone H2A.2 of butyrate-treated Friend erythroleukaemic cells. The histone H2A.2 fraction was isolated by RP-HPLC. CE conditions as in Fig. 3. Abbreviations used were the same as for Fig. 9.

analytical and semipreparative-scale isolation of histone H2A variants, acetylated H2A variants and acetylated H4 histones. So far, no other chromatographic procedure has achieved such an excellent resolution of core histone variants and modified core histone proteins. It thus provides an important alternative to the time-consuming conventional ion-exchange chromatography.

Acknowledgments

This work was supported by FWF Grant S601. We are very grateful to R. Berberich, A. Devich and Dr. M. Rittinger for their excellent technical assistance.

References

[1] A.P. Wolffe, Cell, 77 (1994) 13.
[2] D. Doenecke and D. Gallwitz, Mol. Cell. Biochem., 44 (1982) 113.
[3] R. Reeves, Biochim. Biophys. Acta, 782 (1984) 343.
[4] A. Csordas, Biochem. J., 265 (1990) 23.
[5] B.M. Turner, J. Cell Sci., 99 (1991) 13.
[6] T. Schlake, D. Klehr-Wirth, M. Yoshida, T. Beppu and J. Bode, Biochemistry, 33 (1994) 4197.
[7] V.G. Allfrey, in L. Goldstein and D.M. Prescott (Editors), Cell Biology: a Comprehensive Treatise, Academic Press, London, 1980, vol. 3, p. 347.
[8] P. Loidl, FEBS Lett., 227 (1988) 91.
[9] T.A. Langan, J. Biol. Chem., 257 (1982) 14835.
[10] M. Couppez, A. Martin-Ponthieu and P. Sautiere, J. Biol. Chem., 262 (1987) 2854.
[11] K.W. Marvin, P. Yau and E.M. Bradbury, J. Biol. Chem., 265 (1990) 19839.
[12] A.J. Alpert, J. Chromatogr., 499 (1990) 177.
[13] B.-Y. Zhu, C.T. Mant and R.S. Hodges, J. Chromatogr., 548 (1991) 13.
[14] B.-Y. Zhu, C.T. Mant and R.S. Hodges, J. Chromatogr., 594 (1992) 75.
[15] W. Helliger, H. Lindner, O. Grübl-Knosp and B. Puschendorf, Biochem. J., 288 (1992) 747.
[16] H. Lindner, W. Helliger and B. Puschendorf, J. Chromatogr, 357 (1986) 301.
[17] H. Lindner, W. Helliger and B. Puschendorf, Anal. Biochem., 158 (1986) 424.
[18] W. Helliger, H. Lindner, S. Hauptlorenz and B. Puschendorf, Biochem. J., 255 (1988) 23.
[19] H. Lindner, W. Helliger and B. Puschendorf, J. Chromatogr, 450 (1988) 309.
[20] H. Lindner and W. Helliger, Chromatographia, 30 (1990) 518.
[21] H. Lindner, W. Helliger, A. Dirschlmayer, M. Jaquemar and B. Puschendorf, Biochem. J., 283 (1992) 467.
[22] E.P. Candido, R. Reeves and J.R. Davies, Cell, 14 (1978) 105.
[23] G.W. Grove and A. Zweidler, Biochemistry, 23 (1984) 4436.
[24] T. Hayashi, Y. Ohe, H. Hayashi and K. Iwai, J. Biochem., 88 (1980) 27.