Purification and characterization of the cytoplasmic histone acetyltransferase B of maize embryos

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Abstract From a soluble cellular fraction of maize embryos we purified to apparent homogeneity a cytoplasmic histone acetyltransferase, which matches all criteria for a B-type enzyme. Using 8 chromatographic steps, we achieved a 6700-fold purification of an enzymatically active protein with a molecular weight of ~90 kDa. Under denaturing conditions the protein split into 2 components which migrated at 45 and 50 kDa in SDS-PAGE, suggesting that the native enzyme is a heterodimer. The purified enzyme was characterized in terms of physicochemical and kinetic properties, and substrate specificity. It was specific for histone H4, leading to acetylation of non-acetylated H4 subspecies into the di-acetylated state in vitro. Its activity was coincident with the intensity of DNA replication in meristematic cells during embryo germination. We established an electrophoretic system under non-denaturing conditions for detection of enzyme activity within the gel matrix; in combination with second dimension SDS-PAGE the procedure allowed the unambiguous identification of histone acetyltransferase, even in crude enzyme preparations.

Key words: Chromatin; Histone acetylation; Histone acetyltransferase; Transcription; DNA replication; Maize

1. Introduction

The core histones of eukaryotic cells are reversibly acetylated at the ε-amino group of distinct lysines in the flexible, basic N-terminal protein tail [1]. The functions of this post-translational modification are still unclear, but different levels and patterns of acetylation have been correlated with various biological processes, like histone deposition during DNA replication, histone replacement during differentiation, gene activation or gene silencing [2-11]. The dynamic equilibrium of acetylation is maintained by two enzymes, histone acetyltransferase and histone deacetylase. Both activities exist as multiple enzyme forms that differ with respect to intracellular location, substrate specificity, and activity during the cell cycle and differentiation [12-16].

A promising approach for understanding the functions of histone acetylation is the unraveling of the structure of the enzymes involved and their encoding genes. Until now neither histone acetyltransferases nor deacetylases have been purified to homogeneity in such a way as to allow a direct link of enzyme activity with a purified protein. Recently, a genetic approach has led to the identification of a putative H4 histone acetyltransferase of yeast with partial sequence homology to N-terminal acetyltransferases [17]. Characterization and purification of histone acetyltransferases and deacetylases has a high priority in the chromatin research field.

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We have characterized and purified histone acetyltransferases and deacetylases in germinating maize embryos, a source particularly rich in these enzymes. Histone acetyltransferases can be classified into nuclear A-type enzymes (specific for core histones assembled in nucleosomes) and cytoplasmic B-type activities (specific for free core histones, mainly newly synthesized H4). Histone acetyltransferases have been studied and partially purified from a variety of cells and organisms (e.g. [14,18-34]). However, results are difficult to compare since the reports differ with respect to isolation of enzymes, assay conditions, intracellular localization, and therefore classification into A- or B-type enzymes.

After characterization of maize histone acetyltransferase forms [14-16] we purified the cytoplasmic histone acetyltransferase B of germinating embryos by combining cellular fractionation, multiple chromatographic steps, and an electrophoretic activity gel assay. We achieved a 6700-fold purification of the enzyme, a 90 kDa protein, that is specific for histone H4 and leads to di-acetylation of purified non-acetylated H4 subspecies in vitro.

2. Materials and methods

2.1. Plant material

Maize seeds (*Zea mays*, strain Cuzco) were germinated in darkness for 72 h on cotton layers soaked with water at 28°C. Endosperm-free seedlings were harvested into liquid nitrogen. Cellular fractionation was performed as described [16,35]. Frozen tissue (1 kg) was ground to powder in an Ika grinding machine and quickly suspended in 4 l of precooled solubilization buffer (20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM KCl, 0.25 M sucrose, 0.25% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol). The mixture was stirred until the temperature was raised to −10°C, filtered through 200 and 100 μm (pore size) nylon membranes and centrifuged at 10000×*g* for 15 min at 0°C. The supernatant mainly contains soluble cytoplasmic proteins. To solubilize cytoplasmic histone acetyltransferase activity quantitatively, a large amount of solubilization medium was necessary (4 ml per g wet tissue).

2.2. Protein purification

2.2.1. DEAE-Sepharose batch chromatography. We started the purification of histone acetyltransferase B from 6 kg of maize embryos. Therefore, DEAE-Sepharose batch chromatography was performed 6 times, each time using 1 kg of embryos: 4.5 l of the soluble cytoplasmic portion (equivalent to 1 kg) was incubated with 500 ml of DEAE-Sepharose Fast Flow (Pharmacia Biosystems, Uppsala, Sweden) at 4°C under gentle shaking for 8 h. After washing the gel with 2 l of buffer B (15 mM Tris-HCl, pH 8.5, 10 mM NH_4Cl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol), proteins were eluted under weak suction with a stepwise NH_4Cl gradient using 2 l of 0.1 M NH_4Cl in buffer B and then 2 l of 0.5 M NH_4Cl in buffer B. Batch fractions of 250 ml were collected and assayed for histone acetyltransferase activity. Eluates containing enzyme activity (batches 2–5 of the 0.5 M eluate) were pooled and dialyzed against buffer B.

2.2.2. DEAE-Sepharose column chromatography. Two liters of pooled and dialyzed eluates (2 batch-DEAE elutions) was applied to a DEAE-Sepharose Fast Flow column (5×50 cm; 200 ml of gel),

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Table 1
Purification of histone acetyltransferase B by different chromatographic steps

Purification step	Specific activity (μU/mg protein)	Purification (-fold)
Soluble cytoplasm	0.85	1
DEAE batch pool	7.7	9
DEAE-Sepharose	27.0	32
Poly-lysine agarose	109	128
Phenyl Sepharose	269	316
Histone agarose	491	574
Superdex S-200	647	760
Arginine Sepharose	3000	3480
Resource Q	5700	6730

Aliquots of the soluble cytoplasmic fraction, the DEAE batch pool, and peak fractions of each chromatography were assayed for enzyme activity (see Section 2) and protein content [36]. Specific enzyme activity is expressed as μU/mg protein.

equilibrated with buffer B. The column was washed with 3 column volumes of buffer B at a flow rate of 4 ml/min. Proteins were eluted with a linear gradient (1 l) from 10 mM to 0.5 M NH_4Cl in buffer B. Fractions of 20 ml were collected and assayed for histone acetyltransferase activity. Fractions with maximum activity were pooled and dialyzed against buffer B.

2.2.3. Poly-lysine agarose chromatography. The dialyzed pool of 3 DEAE-Sepharose chromatographies was applied to a poly-lysine agarose (high molecular weight, Sigma Chem., St. Louis, MO, USA) column (2.5×10 cm; 50 ml), equilibrated with buffer B. Flow rate was 48 ml/h. Proteins were eluted with 450 ml of a linear gradient from 10 mM to 1 M NH_4Cl in buffer B. Fractions (9 ml) with maximum activity were pooled, concentrated to a final volume of 35 ml using Amicon Centriprep-30 and dialyzed against 0.8 M NH_4Cl in buffer B.

2.2.4. Hydrophobic interaction chromatography (phenyl Sepharose). The poly-lysine agarose concentrate was applied to a phenyl Sepharose (Pharmacia) column (1.5×20 cm; 20 ml) equilibrated with 0.8 M NH_4Cl in buffer B. Flow rate was 14 ml/h. Proteins were eluted with an NH_4Cl step gradient (2.5 column volumes for each step, buffer B) of 0.8 M, 0.5 M and 10 mM containing 2% (v/v) Triton X-100. Enzyme activity eluted with 2% Triton X-100. Fractions (4 ml) with maximum activity were pooled for histone agarose chromatography.

2.2.5. Histone agarose chromatography. The phenyl Sepharose pool (20 ml) was applied to a histone agarose (Sigma) column (1.5×20 cm; 20 ml; equilibrated with buffer B) at a flow rate of 15 ml/h. Elution was performed with 160 ml of a linear gradient from 10 mM to 0.8 M NH_4Cl in buffer B. Fractions (4 ml) with maximum activity were pooled and concentrated to a final volume of 2 ml using Amicon Centriprep-30.

2.2.6. Size exclusion chromatography (Superdex S-200). Histone agarose concentrate (1 ml) was applied to a Superdex S-200 FPLC column (Pharmacia 2.5×100 cm; 120 ml), equilibrated with 0.2 M NH_4Cl in buffer B. The flow rate was 1 ml/min. Fractions (2.4 ml) with maximum activity of 2 chromatographies were pooled and dialyzed against buffer B.

2.2.7. Arginine Sepharose chromatography. Immediately after size exclusion chromatography the dialyzed enzyme pool was applied to an arginine Sepharose (Pharmacia) column (1×10 cm; 10 ml), equilibrated with buffer B at a flow rate of 15 ml/h. Proteins were eluted with a linear gradient (45 ml) from 10 mM to 0.5 M NH_4Cl in buffer B. Fractions (1.5 ml) with maximum activity were pooled and dialyzed against buffer B.

2.2.8. Resource Q chromatography. The dialyzed enzyme fractions after arginine Sepharose chromatography were applied to a Resource Q FPLC column (Pharmacia; 3×0.4 cm; 1 ml), equilibrated with buffer B. Loading/elution of proteins was performed at a flow rate of 1 ml/min with 40 ml of a linear gradient from 10 mM to 0.5 M NH_4Cl in buffer B. Fractions of 0.75 ml were collected and assayed for histone acetyltransferase activity.

2.3. Histone acetyltransferase assay

Histone acetyltransferase activity was assayed with chicken erythrocyte core histones as substrate [16]. Samples of 100 μl were incubated with 120 μg of histone and 0.05 μCi of [1-^{14}C]acetyl-CoA (ICN Biomedicals, Costa Mesa, CA; 55 mCi/mmol) in a final volume of 125 μl for 20 min at 37°C. An aliquot of 100 μl of the reaction mixture was collected onto glass fiber filter (Whatman GF/F). Filters were air-dried and then submersed in ice-cold 25% (w/v) trichloroacetic acid. After 20 min filters were washed twice with 25% trichloroacetic acid, once with ethanol, once with ethanol:ethyl ether (1:1, v/v) and finally with ethyl ether. After drying filters for 10 min at 70°C, radioactivity was measured by liquid scintillation spectrophotometry.

For the modified histone acetyltransferase assay after polyacrylamide gel electrophoresis under non-denaturing conditions, each gel piece was suspended in 150 μl of buffer A (15 mM Tris-HCl, pH 8.5, 0.2 M NH_4Cl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol) and incubated with 240 μg of histone substrate and 0.1 μCi of [1-^{14}C]acetyl-CoA in a final volume of 200 μl for 3 h at 37°C under permanent shaking. Aliquots of 150 μl of the reaction mixture were removed and the reaction was stopped by addition of 15% trichloroacetic acid. This mixture was incubated on ice for 15 min, collected onto glass fiber filters (Whatman GF/F) under suction and filters were washed three times with 25% (w/v) trichloroacetic acid, three times with ethanol, once with a mixture of ethanol:ethyl ether (1:1, v/v) and finally with ethyl ether. After drying filters for 10 min at 70°C, radioactivity was measured by liquid scintillation spectrophotometry. Protein determination was done as described [36].

2.4. Specificity of histone acetyltransferase B

To determine the substrate specificity of histone acetyltransferase B, the enzyme assay was performed as described [14]. Aliquots of 250 μl of enzyme containing fractions, 125 μg of chicken erythrocyte core histones, and 0.1 μCi of [1-^{14}C]acetyl-CoA were incubated in a final volume of 280 μl for 20 min at 37°C. After incubation, 26 μl of a solution of 2.5 M NaCl/0.5 M Tris-HCl (pH 8.0; final concentration of 0.2 M NaCl) was added. Histones were precipitated from this solution with novobiocin (1 mg/ml) as described [37]. Precipitated histones were recovered by centrifugation, washed with cold ethanol and dried under vacuum. Proteins were analyzed by SDS-15% PAGE [38] and incorporation of radiolabel into histones was detected by fluorography as described [39] using preflashed Amersham Hyperfilm-MP.

To determine the level of H4 acetylation introduced by purified histone acetyltransferase B, we first purified H4 by sulfopropyl Sephadex C-25 chromatography to obtain acetylated subspecies of H4 [40]; the non-acetylated H4 subspecies was incubated with purified histone acetyltransferase B and 0.75 μCi of [1-^{14}C]acetyl-CoA in a final volume of 280 μl for 30 min at 37°C in vitro. After incubation, precipitated H4 was washed twice with ethanol, dried and loaded onto acid-urea-Triton polyacrylamide gels [41] with subsequent fluorography [39].

2.5. Polyacrylamide gel electrophoresis under non-denaturing conditions

10% polyacrylamide gels were prepared [38] except that SDS was omitted. An equal volume of sample buffer (0.1 M Tris-HCl, pH 8.5, 10% glycerol) was added to histone acetyltransferase B preparations of different stages of purification. Electrophoresis was done at 125 V (constant) for 3 h at 4°C (running buffer; 25 mM Tris-HCl, 190 mM glycine, pH 8.5). After electrophoresis the gel lane was vertically cut into 2 halves. One half was sliced into 0.5 cm pieces from top to end and each gel piece was assayed for enzyme activity as described above.

For second dimension SDS-PAGE [38] the remaining half lane of the first dimension gel was incubated in SDS sample buffer (62 mM Tris-HCl, pH 6.8, 0.1 M SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercap-

toethanol) for 5 min at 25°C. The gel lane was then placed onto an SDS-10% polyacrylamide gel with an agarose bridge (1% (w/v) agarose, 125 mM Tris/phosphate, pH 6.8, 0.1% (w/v) SDS). Running conditions were: 5 min at 35 V, 10 min at 55 V and finally 100 V constant. Silver staining of proteins was done as described [42].

3. Results

3.1. Solubilization of histone acetyltransferase B

In a set of experiments we established conditions for the selective solubilization of histone acetyltransferase B from maize embryo tissue. When buffered salt solutions (up to 500 mM NH_4Cl or NaCl) were used as a first step to extract the B-type enzyme from crude tissue homogenates, the resulting preparations were a mixture of B-type and nuclear A-type enzymes; furthermore, the activity of histone acetyltransferase B was extremely unstable, probably due to co-extraction of proteolytic activities. We therefore adopted an established plant chromatin preparation procedure for our purposes; the plant material, homogenized in liquid nitrogen, was suspended in low salt buffer containing 40% glycerol, keeping the temperature down to lower than −10°C during the solubilization period. This was a crucial step of essential importance for further purification. The homogenate was then centrifuged and the high-speed supernatant was used as the enzyme source. Under these conditions the B-enzyme was stable and nuclear A-type histone acetyltransferases were not at all extracted. This is important because contaminating nuclear histone acetyltransferases were difficult to separate from the cytoplasmic B-enzyme in subsequent chromatographic steps. However, for quantitative solubilization of histone acetyltransferase B, large amounts of solubilization buffer (4 ml per g of wet tissue) were necessary. Since we started our final purification with 6 kg of embryos (wet weight), we ended up

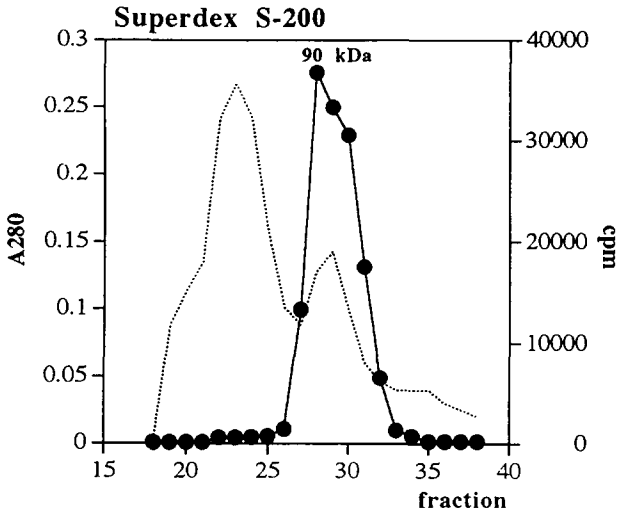


Fig. 1. Gel filtration chromatography for determination of the molecular weight of maize histone acetyltransferase B. Histone acetyltransferase B was purified by a series of chromatographic steps (DEAE-Sepharose batch, DEAE-Sepharose fast flow, poly-lysine agarose, phenyl Sepharose, histone agarose) from maize soluble fraction. Concentrated peak fractions of the histone agarose chromatography were applied to a Superdex S-200 FPLC column. Fractions of 2.4 ml were collected. Elution volume of the maximum enzyme activity corresponds to 90 kDa. Enzyme activity (●) was measured in the standard assay and is expressed as cpm. Protein content (...) was recorded by absorption at 280 nm.

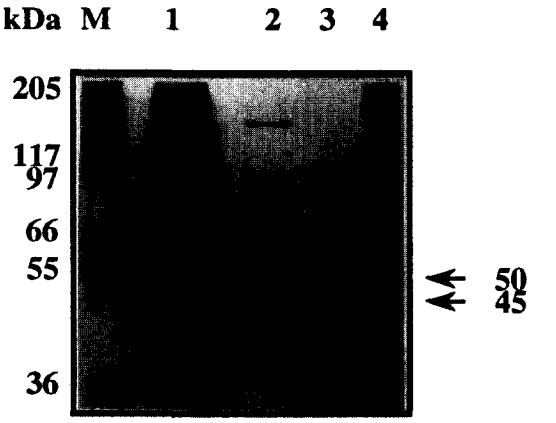


Fig. 2. SDS-PAGE of proteins at different steps of histone acetyltransferase B purification. Peak fractions of phenyl Sepharose (lane 1), histone agarose (lane 2), arginine Sepharose (lane 3), and Resource Q (lane 4) were subjected to SDS-10% PAGE and silver stained. Molecular weight markers are indicated (kDa).

with a total of 27 l of enzyme containing solution. This volume was inappropriate for further chromatographies. We therefore performed the first DEAE-Sepharose as a batch chromatography, incubating aliquots of the sample with DEAE-Sepharose, which was then washed in a funnel with buffered salt batches in a stepwise manner. During this batch chromatography the glycerol concentration was lowered from 40% to 10% and the volume of the sample was decreased. The 500 mM batch (containing the enzyme) was dialyzed and used as starting material for column chromatographies.

3.2. Purification of histone acetyltransferase B by a series of chromatographic steps

The DEAE batch was subjected to 7 consecutive column chromatographies using conventional or FPLC techniques (not shown as figures). Since our enzyme preparation was not contaminated with nuclear A-type acetyltransferases, the activity eluted in a single peak in all chromatographies. It should be pointed out that enzyme activity bound quantitatively to the employed chromatographic materials, so that no activity could be detected in the wash of the columns. In DEAE-Sepharose fast flow chromatography the B-enzyme eluted at a ionic strength of ~270 mM. Although the sample had already been subjected to stepwise DEAE batch elution before, we achieved further enrichment of the enzyme by elution with the linear salt gradient, as reflected by an increase of specific enzyme activity, rising from 7.7 to 27 μU per mg protein (Table 1). Peak fractions were pooled, dialyzed and applied to a column of poly-lysine agarose. The enzyme exhibited strong affinity for this matrix, as reflected by the high ionic strength (~620 mM) necessary for elution of the activity. This chromatographic step yielded a good separation of B-enzyme from the majority of proteins which eluted between 100 and 450 mM salt. The specific enzyme activity was raised to 109 μU (Table 1). Peak fractions were now dialyzed against high salt buffer (0.8 M NH_4Cl in buffer B) to prepare the enzyme for hydrophobic interaction chromatography on phenyl Sepharose. Elution was performed by stepwise lowering of the ionic strength and final addition of 2% Triton X-100. The enzyme eluted as a sharp peak when 10 mM salt and Triton were applied. The specific enzyme activity increased to 269 μU. An additional advantage of the phenyl Sepharose chro-

matography was that the obligatory dialysis step could be omitted, due to the proper salt concentration (10 mM) for the subsequent chromatography. Peak fractions were subjected to affinity chromatography on histone agarose. A considerable proportion of contaminating proteins did not bind to this matrix. The enzyme eluted at 350 mM salt. Histone agarose chromatography resulted in an increase of the specific activity to 491 μU (Table 1). After concentration of the peak fractions by ultrafiltration, the sample was subjected to Superdex S-200 gel filtration chromatography (Fig. 1). The enzyme eluted in a volume corresponding to a molecular weight of ~90 kDa and was well separated from bulk protein which eluted at higher molecular weights. The resulting specific activity was 647 μU (Table 1). To achieve a further enrichment, we applied dialyzed peak fractions (28 and 29) of the gel filtration chromatography to arginine Sepharose. Approximately 60% of the total protein did not bind to this affinity matrix and was therefore found in the wash. The enzyme activity quantitatively bound and eluted at ~210 mM salt in a broad protein peak. The specific activity of the peak fraction was 3000 μU, demonstrating the very good separation of enzyme from contaminating protein (Table 1). To finally split up the heterogeneous protein peak of the arginine Sepharose chromatography and separate the B-enzyme completely from traces of contaminating protein, we applied the peak fractions of arginine Sepharose chromatography after dialysis to high resolution Resource Q chromatography. The total protein eluted within 2 peaks; the first peak eluting at ~300 mM contained the enzyme activity. The peak fraction had a specific enzyme activity of 5700 μU. This represented a 6700-fold purification of the B-enzyme in relation to the soluble cytoplasmic fraction which served as the starting material for purification (Table 1). If one considers that the soluble cytoplasmic fraction is roughly equivalent to 30% of the total protein of the tissue homogenate, our purification strategy achieved a purification of more than 20 000-fold. In a separate small-scale purification experiment in which we used the identical combination of chromatographic steps we applied the Resource Q peak fraction to a Superdex S-75 FPLC size exclusion column. The highly purified enzyme activity again eluted in a fraction corresponding to a molecular weight of 90 kDa (result not shown).

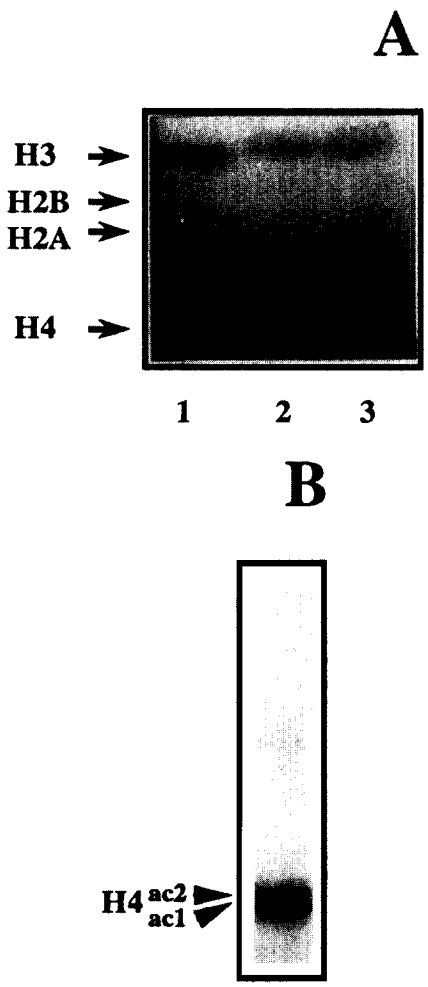


Fig. 3. Specificity of purified maize histone acetyltransferase B. A: Highly purified enzyme preparations (aliquots of peak fractions of 3 independent Resource Q chromatographies; lanes 1–3) were incubated with total core histones in the presence of [^{14}C]acetyl-CoA in vitro. After incubation histones were precipitated and electrophoresed in an SDS-15% polyacrylamide gel with subsequent fluorography. Positions of core histone species are indicated. B: Purified, non-acetylated H4 subspecies was incubated with purified histone acetyltransferase B (peak fraction of Resource Q chromatography) in the presence of [^{14}C]acetyl-CoA in vitro. After incubation, H4 was electrophoresed in an acid-urea-Triton polyacrylamide gel with subsequent fluorography. Positions of mono- and di-acetylated H4 subspecies are indicated.

The peak fraction of the final Resource Q chromatography was subjected to SDS-PAGE. Two protein bands were visible after silver staining, at molecular weights of 45 and 50 kDa (Fig. 2). No other bands were present and the lack of a 90 kDa band indicated that the B-enzyme split into 2 components under denaturing electrophoretic conditions.

We also tested a number of additional chromatographic materials which turned out not to bind histone acetyltransferase B and therefore were useless for our purification strategy. Among those resins were DNA agarose (double-stranded, single-stranded), heparin Sepharose, CoA agarose, as well as various protein binding dye matrices (results not shown).

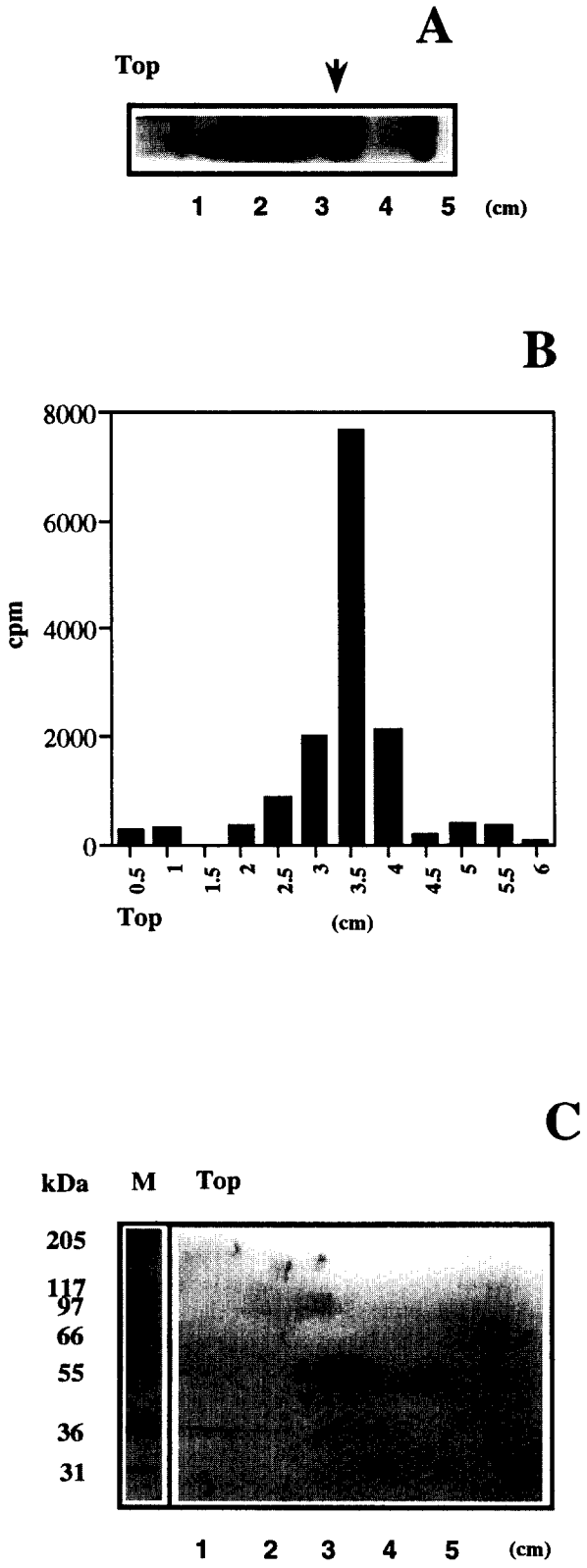
3.3. *Physicochemical/kinetic properties and substrate specificity of purified histone acetyltransferase B*

The purified B-enzyme was used to determine enzymatic parameters. The enzyme had a pH optimum between 8.2 and 8.5. Optimum temperature was 37°C. The enzyme was inhibited by buffered solutions of 5 mM of zinc, copper and iron. It was unaffected by low concentrations (up to 4%) of detergents, like Triton X-100 and Brij 35. It was irreversibly inactivated by 2% ethanol, isopropanol, or dimethyl sulfoxide. Kinetic experiments revealed a k_M value for core histones of 25 μmol/l and 9 μmol/l for acetyl-CoA.

To test the substrate specificity of the purified enzyme, we incubated the Resource Q peak fraction with erythrocyte core histones and [^{14}C]acetyl-CoA. After incubation histones were subjected to SDS-PAGE and fluorography. Fig. 3A shows that the enzyme was highly specific for H4. More than 95% of the label was present in H4, only <5% was in H3. Since the putative function of the B-type enzyme is acetylation of newly synthesized H4, we incubated the pure enzyme with purified non-acetylated H4 subspecies and [^{14}C]acetyl-CoA, in order to check the level of acetylation the enzyme introduces in vitro. Fig. 3B shows an acid-urea-Triton gel of the resulting H4 products. Two labeled bands were visible, corresponding to

mono- and di-acetylated H4 subspecies, showing that the B-enzyme catalyzed di-acetylation of H4.

To check whether the activity of histone acetyltransferase B correlated with DNA replication and histone synthesis, we measured histone acetyltransferase B during the first, partially synchronous cell cycle of maize embryo meristematic tissue [43]. For this purpose we performed small-scale experiments to quantitate the enzyme peaks after DEAE-Sepharose chromatography at different timepoints of germination. As already shown in a similar study from our laboratory [15], histone acetyltransferase B activity closely correlated with DNA replication around 30 h after the start of embryo germination (result not shown).



3.4. Electrophoretic identification of proteins with histone acetyltransferase activity under non-denaturing conditions

To detect enzyme activity and the corresponding protein bands of histone acetyltransferases in polyacrylamide gels at any stage of purification we investigated different activity gel methods. Since maize histone acetyltransferase B lost its catalytic activity after exposure to SDS, we made use of gel electrophoresis under non-denaturing conditions. The samples with enzymatic activity were run in two parallel lanes of a 10% polyacrylamide slab gel. After electrophoresis, one lane was stained (Fig. 4A), a parallel lane was cut into 2 halves; one half was sliced for detection of enzyme activity within the gel (Fig. 4B), the other half was put on top of an SDS-10% polyacrylamide gel for resolution in the second dimension (Fig. 4C). Fig. 4 shows results using a fraction with high enzyme activity of the Superdex S-200 chromatography. The sample was separated into discrete bands in the first dimension under non-denaturing conditions. Maximum enzyme activity was measured in the gel at 3–4 cm of migration (Fig. 4B). The second dimension SDS gel allowed identification of proteins with defined molecular weights in those regions of the first dimension gel where enzyme activity had been detected. It can be seen that the 2 spots of 45 and 50 kDa were present in exactly the region of the first dimension gel that contained histone acetyltransferase B activity. Experiments with partially purified maize nuclear A-type acetyltransferases revealed that the method is also suitable for analysis of other histone acetyltransferase forms (results not shown).

4. Discussion

For more than 30 years posttranslational acetylation of ε-amino groups of N-terminal lysine residues of core histones had been recognized [1]. However, a detailed understanding of the functions of this modification has not yet emerged. As already suggested by previous results from our laboratory [3,44], it now more and more turns out that the level of acetylation per se is less important for regulation of nuclear processes than acetylation or deacetylation of certain lysine residues in a site-specific manner. This was demonstrated by use of site-specific antibodies against ε-acetyl-lysines of H4 [45,46], as well as recently by a genetic approach in yeast; it was shown that replacement of the 4 acetylatable H4 lysines

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Fig. 4. Electrophoretic analysis (first and second dimension) of samples containing histone acetyltransferase B and determination of enzymatic activity in a gel activity assay. A: An enzyme preparation (maximum activity fraction after Superdex S-200 chromatography) was subjected to 10% PAGE under non-denaturing conditions. After electrophoresis a parallel lane was cut into 2 halves. B: One half was sliced and enzyme activity determined in the gel slices. C: The second half of the lane was put on top of a second dimension SDS-10% polyacrylamide slab gel. Gels were stained with silver. Marker proteins (kDa) were co-electrophoresed in the second dimension gel. Arrows mark the position of the 45 and 50 kDa proteins.

by glutamines resulted in delay of cell cycle progression in the G_2 period and mitosis [47]. Insertion of just one lysine residue was sufficient for normal G_2/mitosis progression. Interestingly, lysine could not be substituted by arginine, so a simple charge effect can be ruled out. The mutant in which lysines were replaced by glutamines also had a shortening of the G_1 period; however, the G_1 shortening did not correlate with the G_2/mitosis phenotype, because substitution of lysine-16 by arginine compensated this defect [47]. Such studies demonstrate that acetylation of each lysine residue has a distinct function in epigenetic signalling in chromatin.

If one assumes site-specific functions of acetylation, the enzymes for acetylation and deacetylation must act in a highly specific and coordinated way. For the understanding of the regulatory role of specific acetyl-lysines we need information on histone acetyltransferases and deacetylases, in particular on the DNA sequence of the encoding genes. The purification and molecular characterization of histone acetyltransferases/deacetylases and their encoding genes will enable us to study site specificity, subcellular and tissue distribution during the cell cycle and differentiation, as well as finally the functional consequences of acetylation on development in genetically manipulated organisms. We therefore focused our attention on the identification and characterization of enzymes involved in acetylation in different organisms [14-16,30,31,48] and on the purification of the cytoplasmic histone acetyltransferase B from maize embryos.

In germinating maize embryos there are 3 histone acetyltransferases, a cytoplasmic B-enzyme and 2 nuclear A-forms [14-16]. For several reasons we chose the cytoplasmic B-enzyme for purification. The A-type enzymes have to be extracted from either isolated nuclei or total cellular homogenate with salt buffers. Therefore any preparation will contain a mixture of at least 2 nuclear enzymes which we found difficult to separate quantitatively by chromatographic methods. On the other hand, a simple cellular fractionation, which is routinely used for plant chromatin preparation, yielded a cytoplasmic fraction that only contained the B-enzyme without even traces of nuclear acetyltransferases. Furthermore, it is known that the B-type enzyme is specific for free histone H4. This fact allows a faithful enzymatic assay in contrast to nuclear enzyme forms that are specific for histones packaged in nucleosomes, but it also sets a criterion for the unambiguous identification of the enzyme type. For further studies with the purified enzyme, but also for functional studies at a genetic level, it may be helpful that for the cytoplasmic B-enzyme a specific function is known, namely the acetylation of newly synthesized H4 prior to chromatin assembly during DNA replication [49,50]. It also seems reasonable to assume that the DNA sequence of the B-enzyme encoding gene will be a sufficient molecular tool to isolate genes encoding nuclear A-type enzymes.

In the initial stage of our investigation on histone acetylation enzymes we screened a panel of organisms (yeast, *Physarum*, plant embryos, rat liver, chicken reticulocytes) for activity and stability of acetyltransferases. We found maize embryo cells to be particularly rich in these enzymes, which may partly be due to the huge amount of DNA in this organism. Moreover, maize embryo cells are distinguished from other cells in that nuclear A-type enzymes are tightly bound to chromatin and do not leak off the nuclei during tissue homogenization.

There have been numerous attempts to characterize and purify histone acetyltransferases in a wide variety of organisms (e.g. [18-20,23,24,29]). The k_M value for core histones (25 μM) that we determined for maize histone acetyltransferase B was in the range of previously published values, which varied between 1 and 20 μM [18,20,23,25]. Most of the previous purification studies were performed with nuclear A-enzymes, although it is not always clear whether a homogeneous enzyme form (A- or B-type) was investigated; there is one report in the literature that definitely dealt with the partial purification of a B-type histone acetyltransferase from *Drosophila* [20]. Most of the studies in the literature ended up with partial purification as judged from the Coomassie blue stained SDS-polyacrylamide slab gels.

When we started this investigation, no histone acetyltransferase had been purified to homogeneity in such a way that a clearcut correlation of enzymatic activity with certain protein bands on the final SDS gel could be made. Recently, an activity gel assay has been introduced that should allow the renaturation of histone acetyltransferase after normal SDS-PAGE with subsequent determination of enzyme activity in the gel matrix [34]; using this assay the authors have identified a 55 kDa protein in macronuclear extracts of *Tetrahymena*. The molecular weight of the native enzyme was determined to be 220 kDa by gel filtration chromatography, indicating that the 55 kDa protein might be a subunit of a higher molecular weight protein [34]. This is similar to the situation in maize embryos, since we also found subunit proteins in this molecular weight range (45 and 50 kDa), which obviously build up a higher molecular weight protein. Porcine liver histone acetyltransferases [29] have been reported to consist of 62 and 45 kDa protein components (type I) or 50 and 40 kD proteins (type II). Unfortunately, histone acetyltransferase B of maize is more fragile than the *Tetrahymena* enzyme [34], since we had difficulties restoring enzymatic activity once the enzyme had been treated with SDS. Furthermore, the enzyme was sensitive to ethanol or isopropanol. When we performed the SDS gel activity assay [34] we observed non-enzymatic, chemical acetylation of proteins [51], even after inclusion of 200 mM salt, when we loaded crude or purified enzyme preparations on the gel. We developed an electrophoretic system that combines electrophoresis in the absence of SDS under non-denaturing conditions, allowing detection of enzyme activity in the gel, with a second dimension SDS-PAGE. This represents an alternative possibility for identification of enzymatically active proteins in complex or partially purified histone acetyltransferase fractions.

During the preparation of this article the identification of a yeast gene encoding for a histone acetyltransferase was reported [17]. By screening a collection of yeast temperature-sensitive mutants for histone acetyltransferase activity the authors picked up a mutation that affected acetylation of an N-terminal H4 peptide. The mutant did not exhibit any obvious phenotype. The cloned *HAT1* gene encoded a protein of 374 amino acids, corresponding to a molecular weight of 44 kDa, with a large number of phenylalanines. When the HAT1 protein was expressed in *E. coli*, the product had histone acetyltransferase activity. Homology search revealed that *HAT1* shared a bipartite consensus sequence with previously identified N-terminal acetyltransferases. It still remains unclear whether the yeast HAT1 enzyme represents a B-type enzyme, because the substrate specificity was only tested

with N-terminal substrate peptides of H4 and H3, but neither with H2A and H2B, nor with a complete set of core histones in a competitive assay. The enzyme did accept both histones, H4 and H3, as substrate. In contrast, the maize enzyme was highly specific for H4 and fulfils the additional criterion for B-type enzymes, namely acetylation of H4 up to the di-acetylated state. Comparison of cDNA sequence data will soon enable us to identify homologous regions between the yeast and maize acetyltransferases and judge the degree of evolutionary conservation among these proteins.

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References

[1] Allfrey, V.G., Faulkner, R. and Mirsky, A.E. (1964) Proc. Natl. Acad. Sci. USA 51, 786–794.
[2] Doenecke, D. and Gallwitz, D. (1982) Mol. Cell. Biochem. 44, 113–128.
[3] Loidl, P. (1988) FEBS Lett. 227, 91–95.
[4] Turner, B.M. (1991) J. Cell Sci. 99, 13–20.
[5] Bradbury, E.M. (1992) BioEssays 14, 9–16.
[6] Ausio, J. (1992) J. Cell Sci. 102, 1–5.
[7] López-Rodas, G., Brosch, G., Georgieva, E.I., Sendra, R., Franco, L. and Loidl, P. (1993) FEBS Lett. 317, 175–180.
[8] Turner, B.M. (1993) Cell 75, 5–8.
[9] Wolffe, A.P. (1994) Cell 77, 13–16.
[10] Loidl, P. (1994) Chromosoma 103, 441–449.
[11] Brosch, G., Ransom, R., Lechner, T., Walton, J.D. and Loidl, P. (1995) Plant Cell 7, 1941–1950.
[12] Sendra, R., Rodrigo, I., Salvador, M.L. and Franco, L. (1988) Plant Mol. Biol. 11, 857–866.
[13] López-Rodas, G., Tordera, V., Sánchez del Pino, M.M. and Franco, L. (1989) J. Biol. Chem. 264, 19028–19033.
[14] López-Rodas, G., Georgieva, E.I., Sendra, R. and Loidl, P. (1991) J. Biol. Chem. 266, 18745–18750.
[15] Georgieva, E., López-Rodas, G., Sendra, R., Gröbner, P. and Loidl, P. (1991) J. Biol. Chem. 266, 18751–18760.
[16] Grabher, A., Brosch, G., Sendra, R., Lechner, T., Eberharter, A., Georgieva, E.I., López-Rodas, G., Franco, L., Dietrich, H. and Loidl, P. (1994) Biochemistry 33, 14887–14895.
[17] Kleff, S., Andrulis, E.D., Anderson, C.W. and Sternglanz, R. (1995) J. Biol. Chem. 270, 24674–24677.
[18] Belikoff, E., Wong, L.-J. and Alberts, B.M. (1980) J. Biol. Chem. 255, 11448–11453.
[19] Böhm, J., Schlaeger, E.-J. and Knippers, R. (1980) Eur. J. Biochem. 112, 353–362.
[20] Wiegand, R.C. and Brutlag, D.L. (1981) J. Biol. Chem. 256, 4578–4583.
[21] Wiktorowicz, J.E., Campos, K.L. and Bonner, J. (1981) Biochemistry 20, 1464–1467.
[22] Estepa, I. and Pestana, A. (1983) Eur. J. Biochem. 132, 249–254.
[23] Kelner, D.N. and McCarthy, K.S. (1984) J. Biol. Chem. 259, 3413–3419.
[24] Travis, G.H., Colavito-Shepanski, M. and Grunstein, M. (1984) J. Biol. Chem. 259, 14406–14412.
[25] Yukioka, M., Sasaki, S., Qi, S.-L. and Inoue, A. (1984) J. Biol. Chem. 259, 8372–8377.
[26] López-Rodas, G., Perez-Ortin, J.E., Tordera, V., Salvador, M.L. and Franco, L. (1985) Arch. Biochem. Biophys. 239, 184–190.
[27] Sendra, R., Salvador, M.L., López-Rodas, G., Tordera, V. and Franco, L. (1986) Plant Sci. 46, 189–194.
[28] Chicoine, L.G., Richman, R., Cook, R.G., Gorovsky, M.A. and Allis, C.D. (1987) J. Cell Biol. 105, 127–135.
[29] Attisano, L. and Lewis, P.N. (1990) J. Biol. Chem. 265, 3949–3955.
[30] Brosch, G., López-Rodas, G., Golderer, G., Lindner, H., Gröbner, P. and Loidl, P. (1992) Cell Biol. Int. Rep. 16, 1103–1109.
[31] López-Rodas, G., Brosch, G., Golderer, G., Lindner, H., Gröbner, P. and Loidl, P. (1992) FEBS Lett. 296, 82–86.
[32] Mingarro, I., Sendra, R., Salvador, M.L. and Franco, L. (1993) J. Biol. Chem. 268, 13248–13252.
[33] Sobel, R.E., Cook, R.G. and Allis, D.C. (1994) J. Biol. Chem. 269, 18576–18582.
[34] Brownell, J.E. and Allis, C.D. (1995) Proc. Natl. Acad. Sci. USA 92, 6364–6368.
[35] Steinmüller, K. and Apel, K. (1986) Plant Mol. Biol. 7, 87–94.
[36] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[37] Cotten, M., Bresnahan, D., Thompson, S., Sealy, L. and Chalkley, R. (1986) Nucleic Acids Res. 14, 3671–3686.
[38] Laemmli, U.K. (1970) Nature 227, 680–685.
[39] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[40] Couppez, M., Ponthieu, A.M. and Sautiere, P. (1987) J. Biol. Chem. 262, 2854–2860.
[41] Zweidler, A. (1978) Methods Cell Biol. 17, 223–233.
[42] Oshawa, K. and Ebata, N. (1983) Anal. Biochem. 135, 409–415.
[43] Georgieva, E.I., López-Rodas, G., Hittmair, A., Feichtinger, H., Brosch, G. and Loidl, P. (1994) Planta 192, 118–124.
[44] Loidl, P. and Gröbner, P. (1987) Nucleic Acids Res. 15, 8351–8366.
[45] Turner, B.M., Birley, A.J. and Lavender, J. (1992) Cell 69, 375–384.
[46] Jeppesen, P. and Turner, B.M. (1993) Cell 74, 281–289.
[47] Megee, P.C., Morgan, B.A. and Smith, M.M. (1995) Genes Dev. 9, 1716–1727.
[48] Brosch, G., Georgieva, E.I., López-Rodas, G., Lindner, H. and Loidl, P. (1992) J. Biol. Chem. 267, 20561–20564.
[49] Kaufman, P.D., Kobayashi, R., Kessler, N. and Stillman, B. (1995) Cell 81, 1105–1114.
[50] Krude, T. (1995) Curr. Biol. 5, 1232–1234.
[51] Delpech, M., Levy-Favathier, F. and Kruh, J. (1983) Biochimie 65, 291–294.