Purification of histone deacetylase HD1-A of germinating maize embryos

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Abstract **We have purified the soluble nuclear histone deacetylase HD1-A of germinating maize embryos. By a combination of 6 chromatographic steps we achieved a 77 000-fold purification of an enzymatically active protein. Gel filtration chromatography revealed a molecular weight of 45 kDa of the native enzyme and electrophoretic analysis of the purified enzyme by SDS-PAGE resulted in a single band at a molecular weight of 48 kDa, indicating that the enzyme is a monomer protein. When fractions with enzyme activity of different stages of chromatographic purification were subjected to isoelectric focusing, enzyme activity focused at a pH of around 6.4 as measured in an activity gel assay; second dimension SDS-PAGE again revealed a protein spot at a molecular weight of 48 kDa.**

Key words: Chromatin; Histone acetylation; Histone deacetylase; Nucleosome; Nuclear protein; Maize

1. Introduction

Core histones, which are among the most highly conserved proteins in eukaryotic cells, have attracted considerable research interest as important regulators of gene expression [1], apart from their evident structural role in chromatin organization. One of the reasons why these highly conserved, abundant and ubiquitous nuclear proteins are able to fulfil regulatory functions is the fact that they are reversibly modified by a number of posttranslational reactions, such as acetylation, phosphorylation, ADP-ribosylation, ubiquitination and glycosylation [2].

Modification of core histones by acetylation of ε-amino groups of conserved lysine residues in the N-terminal part is a dynamic enzymatic process whose exact biological functions still remain unclear [2–6]. Different levels and patterns of histone acetylation have been correlated with various nuclear processes, like chromatin assembly and histone deposition during DNA replication, histone replacement by primary structure variants during differentiation, or chromatin structural changes during gene activation or repression (e.g. [7–15]). The steady state of histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. These enzyme activities are present as multiple enzyme forms whose activities, properties, intracellular/intranuclear location and substrate specificities are regulated in a complex, cell cycle-related manner [16–22].

Histone deacetylases have been investigated from a variety of organisms [23–27]. In lower eukaryotes and plants these enzymes exist in multiple forms [26,28]. In maize four different histone deacetylases can be distinguished during embryo germination [16,17,20,22,29]; these enzymes differ considerably from each other in terms of substrate specificity, intranuclear location, kinetic properties and posttranslational modification. In yeast a nuclear histone deacetylase could be isolated as a high molecular weight complex (> 350 kDa) at low ionic strength [27]; when the ionic strength was increased to 0.5 M NH_4Cl, the enzyme activity was present in a low molecular weight form of ~150 kDa. The two forms of histone deacetylase differed significantly in their response to the specific deacetylase inhibitor Trichostatin. A similar high molecular weight histone deacetylase complex was also found in chicken erythrocytes [30].

A promising approach for understanding the biological functions of histone acetylation is the analysis of the involved enzymes and their encoding genes. Recently, significant advances have been achieved in this area. A genetic approach was used to identify a B-type histone acetyltransferase of yeast (HAT1) with limited sequence homology to N-terminal acetyltransferases [31]; our laboratory has purified a cytoplasmic histone acetyltransferase B of maize to homogeneity [32]; a nuclear A-type histone acetyltransferase of *Tetrahymena* has been identified as a homolog of the yeast transcriptional coactivator Gcn5p [33]. By utilizing the high affinity of histone deacetylases to cyclic tetrapeptides [21,34], a mammalian histone deacetylase was recently identified [35].

In germinating maize embryos four nuclear histone deacetylases can be distinguished [29]. We have purified the soluble deacetylase HD1-A by six chromatographic steps to apparent homogeneity. The purified protein has a molecular weight of ~48 kDa, as estimated under native (gel filtration chromatography) and denaturing (SDS-PAGE) conditions. The enzymatically active protein could be detected at a pI of ~6.4 after isoelectric focusing. The corresponding pH area of the second dimension SDS gel contained the 48 kDa protein band.

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. The endosperm free seedlings were harvested into liquid nitrogen. Cellular fractionation was performed as described [36]. Frozen tissue (1 kg) was ground to powder in an Ika grinding machine and then quickly suspended in 4 liter 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 and filtered through 200 and 100 μm (pore size) nylon membranes. After centrifugation at 10 000 × *g* for 15 min at 0°C, the supernatant was decanted. The supernatant mainly contains cytoplasmic proteins and also nuclear proteins which are not tightly bound to chromatin. To solubilize histone deacetylase HD1-A activity quantitatively, a high amount of solubilization medium was necessary (4 ml per g wet tissue). For the final purification of HD1-A, the chromatin fraction of a total of 6 kg of embryo tissue was used.

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2.2. Protein purification

2.2.1. Q-Sepharose chromatography. Q-Sepharose chromatography was used as a first step to separate HD1-A. For processing 6 kg of starting material three Q-Sepharose chromatographies were performed: 8 liter of the soluble cytoplasmic portion was batched with 500 ml Q-Sepharose Fast Flow (Pharmacia Biosystems, Uppsala, Sweden), equilibrated with buffer B, for 12 h, washed in a sintered glass filter and filled in an FPLC column (5×30 cm). Elution of bound proteins was performed with 2 liter of a linear gradient from 10 mM to 350 mM NaCl in buffer B at a flow rate of 3 ml/min. Fractions of 20 ml were collected and assayed for histone deacetylase activity. Fractions with high enzyme activity of three chromatographies were pooled, concentrated in an Amicon ultrafiltration cell (molecular weight cut-off 30 kDa) to a final volume of 600 ml and then adjusted to 2 M NaCl in buffer B using a stock solution of 4.5 M NaCl in buffer B.

2.2.2. Hydrophobic interaction chromatography (phenyl-Sepharose). The concentrates of three Q-Sepharose chromatographies were applied onto three phenyl-Sepharose (Pharmacia) columns (3×10 cm; 70 ml) previously equilibrated with 2 M NaCl in buffer B. Flow rate was 0.8 ml/min. Fraction volume was 5 ml. Proteins were eluted with a linear gradient from 2 M NaCl to 10 mM NaCl in buffer B. Fractions with high enzymatic activity were pooled, concentrated using an Amicon ultrafiltration cell (cut-off 30 kDa) to a final volume of 50 ml and dialyzed against buffer B.

2.2.3. Heparin-Sepharose chromatography. Pools of three phenyl-Sepharose chromatographies were loaded onto three heparin-Sepharose columns (Pharmacia; 3×20 ml, 60 ml), equilibrated with buffer B. Proteins were eluted with 150 ml of a linear gradient from 10 mM to 500 mM NaCl in buffer B. Using a flow rate of 1 ml/min, fractions of 4 ml were collected and assayed for histone deacetylase activity. Fractions containing high enzyme activity were pooled and dialyzed against buffer B.

2.2.4. Histone-agarose chromatography. The Heparin-Sepharose pools were applied onto three histone-agarose (Sigma Chem., St. Louis, MO, USA) columns (1.5×20 cm; 15 ml) equilibrated with buffer B at a flow rate of 0.8 ml/min. Elution was performed with 80 ml of a linear gradient from 10 mM to 500 mM NaCl in buffer B; fractions of 3 ml were collected and assayed for histone deacetylase activity. Fractions containing high enzyme activity were pooled and concentrated to a final volume of 1 ml by centrifugation (5000×*g*; 4°C) in an Amicon Centriprep-30.

2.2.5. Size exclusion chromatography (Superdex S-75). Each of the three histone-agarose concentrates was applied onto a Superdex S-75 FPLC column (2.5×100 cm; 120 ml; Pharmacia), equilibrated with 0.2 M NaCl in buffer B. The flow rate was maintained at 1 ml/min, fractions of 1 ml were collected and assayed for histone deacetylase activity. Fractions containing maximum enzyme activity of three chromatographic runs were pooled, concentrated to a final volume of 1 ml by centrifugation (5000×*g*; 4°C) in an Amicon Centriprep-30 and dialyzed against buffer C (20 mM sodium phosphate, pH 8.0, 0.1% (v/v) 2-mercaptoethanol).

2.2.6. Hydroxyapatite chromatography. The dialyzed pool of the three S-75 chromatographies was applied onto two hydroxyapatite Bio-Scale CHT-I (Bio-Rad, Richmond, CA, USA; 2 ml) columns, equilibrated with buffer C. Elution of protein was performed with 30 ml of a linear gradient from 10 mM to 250 mM sodium phosphate. Flow rate was 1 ml/min. Fractions of 1 ml were collected and assayed for histone deacetylase activity.

2.3. Histone deacetylase assay

Histone deacetylase activity was determined as described [26] using [^{3}H]acetate prelabelled chicken reticulocyte histones [37] as substrate. Sample aliquots of 25 µl were mixed with 10 µl of total [^{3}H]acetate prelabelled chicken reticulocyte histones (1.5 mg/ml), resulting in a final concentration of 9 µM. This mixture was incubated at 30°C for 20 min. The reaction was stopped by addition of 36 µl of 1 M HCl/0.4 M acetate and 1 ml ethylacetate. After centrifugation at 10000×*g* for 5 min, an aliquot of 600 µl of the upper phase was counted for radioactivity in 3 ml liquid scintillation cocktail.

For determination of histone deacetylase activity in gel slices after isoelectric focusing, the assay conditions were slightly modified. Each gel piece (0.5×0.5 cm) was put in 150 µl of buffer B, pH 9.2 to 7.9 (to adjust appropriate pH), and incubated with 10 µl of [^{3}H]acetate prelabelled chicken reticulocyte histones as substrate for 1 h at 30°C under permanent shaking.The reaction was stopped by addition of 72 µl of 1 M HCl/0.4 M acetate and the released [^{3}H]acetate was extracted with 0.8 ml ethylacetate by head over head shaking for 1 h at room temperature. After centrifugation at 10000×*g* for 5 min an aliquot of 600 µl of the upper phase was counted for radioactivity in 3 ml liquid scintillation cocktail.

2.4. Protein analysis

SDS-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gels as previously described [38]. Aliquots of histone deacetylase enzyme preparations of different stages of purification were analyzed. Proteins on gels were stained with silver as described [40] with minor modifications. Gels were soaked in 50% methanol for 90 min, incubated in 150 ml of staining solution (0.02 N NaOH, 0.37% NH_4OH and 0.8% $AgNO_3$) for 10 min, washed in distilled water for 10 min and finally developed in 250 ml of a solution containing 0.01% citrate and 0.037% formaldehyde.

2.5. Molecular weight analysis

Estimation of the molecular weight of the native enzyme was performed by gel filtration chromatography. A Superdex S-75 column (2.5×100 cm; 120 ml; Pharmacia) equilibrated with 0.2 M NaCl in buffer B was calibrated with proteins of known molecular weight. The flow rate was maintained at 1 ml/min, fractions of 1.5 ml were collected. Estimation of molecular weight of the purified enzyme under denaturing conditions was performed with SDS-PAGE.

2.6. Isoelectric focusing

Ampholine PAG plates (precast polyacrylamide gels, pH 4.0–7.0, dimension 245×110×1 mm; Pharmacia) were used on a precooled (10°C) Multiphor II electrophoresis unit (Pharmacia). After pre-electrophoresis of the gel for 20 min, an equal volume of sample buffer (0.2 M β-alanine, 30% glycerol) was added to the HD1-A enzyme preparation and 50 µl of sample was applied at 5 mm distance from the cathode (cathode solution: 0.1 M β-alanine; anode solution: 0.1 M glutamic acid in 0.5 M H_3PO_4). Electrophoresis was carried out at 2000 V (const.), 25 mA and 25 W for 1 h 15 min. After electrophoresis the gel lane was vertically cut into two halves. One half was sliced into 0.5 cm pieces from the top to the end and each gel piece was assayed for histone deacetylase activity as described above.

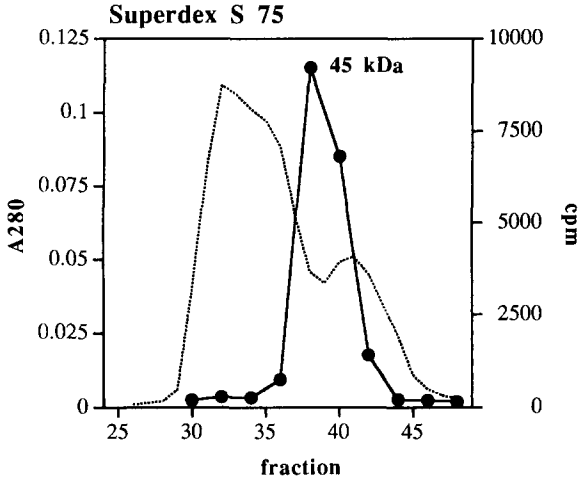

Fig. 1. Superdex S-75 gel filtration chromatography for determination of the molecular weight of maize histone deacetylase HD1-A. Histone deacetylase HD1-A was purified by four consecutive chromatographic steps (Q-Sepharose, phenyl-Sepharose, heparin-Sepharose, histone-agarose) from a soluble fraction of maize embryos at 72 h after the start of germination. Concentrated peak fractions of the histone-agarose chromatography were applied to a Superdex S-75 FPLC column. Fractions of 1.5 ml were collected. Elution volume of the maximum of enzyme activity corresponds to 45 kDa. Enzyme activity (●) was measured in the standard assay and is expressed as cpm. Protein content (···) was recorded by absorption at 280 nm.

For second dimension SDS-PAGE the remaining half (0.5×6 cm) of the lane of the first dimension gel was first incubated in equilibration buffer (0.125 M Tris-HCl, 2% SDS, 10% glycerol, 4.9 mM dithiothreitol, pH 6.8) followed by incubation in equilibration buffer plus 4.5% iodoacetamide for 10 min at room temperature in each case. After washing with running buffer the gel lane was placed onto an SDS-10% polyacrylamide gel. The running conditions of the SDS-PAGE were 1.5 h at 150 V constant.

3. Results

3.1. Purification of histone deacetylase HD1-A by a sequence of chromatographic steps

In germinating maize embryos, four nuclear histone deacetylases are present [29]. The different deacetylases are distinguished by their association with chromatin [22]. The histone deacetylase HD1-A is not associated with chromatin and is therefore readily solubilized and cofractionates with the bulk of cytoplasmic proteins at 72 h after the start of embryo germination. In contrast, histone deacetylases HD1-BI, HD1-BII and HD2 are tightly chromatin bound at this period of germination. Using a well established chromatin isolation procedure [32,36] we purified HD1-A from the soluble fraction of 6 kg (wet weight) of embryo tissue (chromatographies not shown as figures). The soluble protein fraction was first subjected to Q-Sepharose chromatography, where the enzyme activity eluted at a ionic strength of 280 mM. This step resulted in a 185-fold purification as compared to the total soluble protein fraction (Table 1). The pooled peak fractions were adjusted to 2 M NaCl and applied onto a phenyl-Sepharose column. Using a linear salt gradient, the enzyme activity eluted at 1.1 M salt and was well separated from the bulk of contaminating proteins, resulting in a 1820-fold purification (Table 1). After dialysis the pooled peak fractions were subjected to affinity chromatography on heparin-Sepharose. Most of the contaminating proteins were in the wash of the column; bound HD1-A eluted at a ionic strength of 260 mM salt, resulting in a 4910-fold purification. The HD1-A pool was then subjected to histone-agarose chromatography where approximately 50% of the contaminating proteins were in the wash of the column whereas HD1-A was bound to the affinity matrix and eluted at a ionic strength of 230 mM. The purification was 9150-fold after this chromatographic step (Table 1). The concentrated peak fractions were directly loaded onto a Superdex S-75 size exclusion column where the enzyme eluted at an apparent molecular weight of approximately 45 kDa (Fig. 1). Again this chromatography yielded good separation of the enzyme from the bulk of proteins, resulting in a 41 920-fold purification (Table 1). In the final step the peak fractions of the Superdex S-75 chromatography were subjected to hydroxyapatite chromatography where the last traces of contaminating proteins were found in the wash of the column or separated from the bound enzyme activity; HD1-A activity was quantitatively bound to the hydroxyapatite matrix. The enzyme eluted at 190 mM ionic strength, resulting in a final purification of 77 000-fold (Table 1). We analyzed the peak fraction of the hydroxyapatite chromatography on SDS-polyacrylamide slab gels (Fig. 2). The purified sample contained only one protein band; this protein band migrated at an apparent molecular weight of 48 kDa in silver stained gels which is in agreement with the calculated molecular weight of 45 kDa of the gel filtration chromatography.

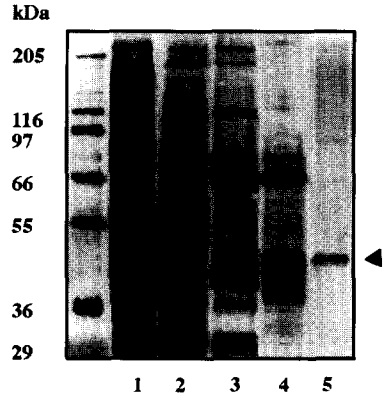

Fig. 2. SDS-PAGE of proteins at different steps of histone deacetylase HD1-A purification. Chromatographic peak fractions of Q-Sepharose (lane 1), phenyl-Sepharose (lane 2), heparin-Sepharose (lane 3), histone-agarose (lane 4), and hydroxyapatite (lane 5) were subjected to SDS-10% PAGE (silver stained). Molecular weights of marker proteins are shown in kDa. Arrow marks the position of HD1-A in lane 5.

3.2. Electrophoretic identification of HD1-A under non-denaturing conditions

In order to identify enzymatically active HD1-A after electrophoresis under non-denaturing conditions, we subjected pooled fractions of the Superdex S-75 gel filtration chromatography to isoelectric focusing between pH 4 and 7. After electrophoresis the isoelectric focusing lane was cut into two halves; one half was sliced into 0.5 cm pieces which were used for determination of enzyme activity (Fig. 3A); the other half of the isoelectric focusing gel lane was put on top of an SDS-polyacrylamide slab gel to separate proteins in the second dimension (Fig. 3B). In the isoelectric focusing gel enzyme

Table 1
Purification of *Zea mays* histone deacetylase HD1-A

Purification step	Protein (mg/ml)	Activity (cpm/ml)×10^{-3}	Specific activity (cpm/mg protein)×10^{-3}	Purification (-fold)
Soluble cytoplasm	35	45.5	1.3	1
Q-Sepharose	1.5	360	240	185
Phenyl-Sepharose	0.3	712	2370	1820
Heparin-Sepharose	0.138	880	6380	4910
Histone-agarose	0.084	1000	11 900	9150
Superdex S-75	0.044	2400	54 500	41 920
Hydroxyapatite	0.015	1500	100 000	76 920

Aliquots of the soluble cellular fraction and the peak fractions of each chromatography were assayed for enzyme activity (see Section 2) and protein content [39].

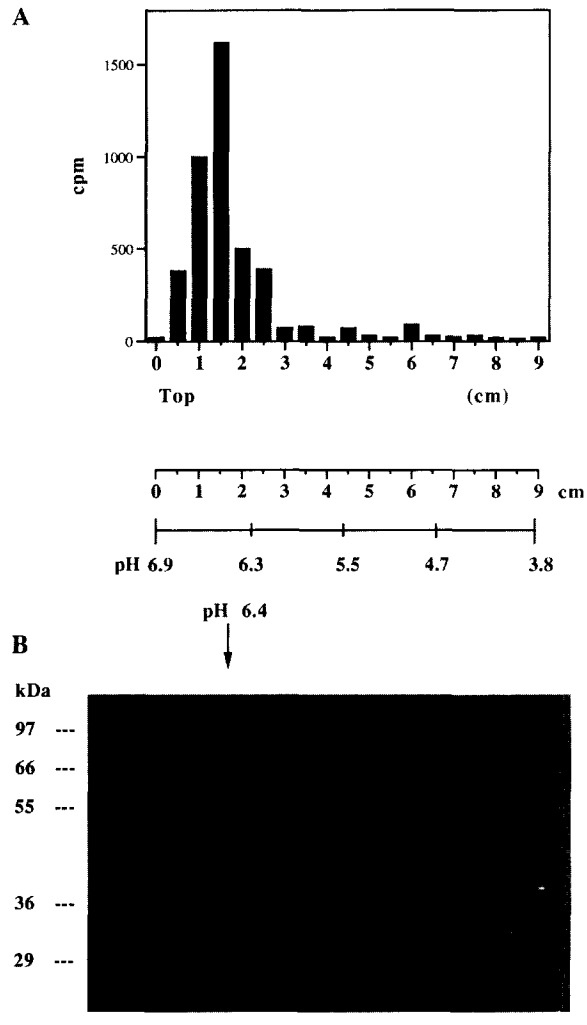

Fig. 3. Electrophoretic analysis (first and second dimension) of samples containing partially purified histone deacetylase HD1-A and determination of enzymatic activity in a gel activity assay. A: An enzyme preparation (pooled fractions after Superdex S-75 chromatography) was subjected to isoelectric focusing in PAG plates (Pharmacia; pH 4–7). After focusing the gel lane was cut into two halves. One half was sliced into 0.5 cm slices and enzyme activity determined in the gel pieces (note that the column at 0 cm corresponds to the slice from 0–0.5 cm migration, column at 0.5 cm corresponds to the slice from 0.5–1.0 cm of migration, etc.); enzyme activity is expressed as cpm. B: The second half of the gel lane was put on top of a second dimension SDS-10% polyacrylamide slab gel. The gel was stained with silver. Marker proteins (kDa) were co-electrophoresed in the second dimension gel. An arrow marks the position of the 48 kDa protein (HD1-A). Ruler (cm) and pH scale correspond to the dimensions of the isoelectric focusing gel.

activity was detected at a pI of ~6.4 (Fig. 3A). The corresponding area in the silver stained second dimension SDS gel contained the 48 kDa protein band (Fig. 3B) which was the only protein band in the highly purified HD1-A preparation after the final hydroxyapatite chromatography (Fig. 2). This result was obtained with partially and highly purified HD1-A samples at different stages of purification (data not shown).

4. Discussion

The flexible N-terminal tails of the core histones allow their positively charged surfaces to interact with DNA or certain domains of regulatory chromosomal proteins. Specific mutations in the amino termini have distinct effects on gene activation, silencing and repression [41]. The amino terminal histone tails can be acetylated at certain lysine residues, resulting in a decrease of the net positive charge on one hand, and in an alteration of their specific binding properties for regulatory proteins on the other hand. A change in the specific acetylation pattern of core histones has therefore been correlated with changes in the transcriptional activity of genes [11,42–44]. Core histone N-termini and their acetylation also play a complex, but still poorly understood role in DNA replication [14,15,45].

For the understanding of the exact function of core histone acetylation we need information on the enzymes which establish and maintain the acetylation state. Recently, purified enzymes and sequences of encoding genes of histone acetyltransferases and deacetylases have become available [31–33,35]. In most experimental systems investigated so far histone acetyltransferases and deacetylases exist as multiple enzyme forms; in pea, three histone deacetylases exist [26]. In maize we could distinguish four histone deacetylases [16,17,20,22,29]. The four histone deacetylases of maize are distinct with respect to their biochemical and kinetic properties [29], to modification by phosphorylation [20], pattern of enzyme activity during the cell cycle and differentiation [16–18], substrate specificity [16,20,29] and subnuclear localization [22]. It is puzzling that despite their different properties, all deacetylases of maize are equally inhibited by different unspecific and specific inhibitors, like butyrate, Trichostatin and cyclic tetrapeptides [21,29]. The fact that multiple deacetylases exist and are subject to complex regulation emphasizes their importance for chromatin structure and function [21,43].

In this study we purified the soluble nuclear histone deacetylase HD1-A of germinating maize embryos. The fact that the apparent molecular weight of the enzyme under non-denaturing conditions (gel filtration chromatography) is identical to the molecular weight calculated from its migration in SDS-polyacrylamide gels, indicates that the deacetylase is a simple monomer protein. In contrast, HD2 from maize embryos exists as a high molecular weight complex with a molecular weight of around 400 kDa that is composed of three highly homologous protein subunits (Brosch, unpublished data). It is extremely unlikely that HD1-A represents a component of the high molecular weight HD2 complex, since its chromatographic properties are completely different from those of the HD2 subunits. Furthermore, we have shown previously that HD1-A is phosphorylated and is not associated with chromatin, irrespective of the stage of embryo germination [20,22]. Evidence for a high molecular weight deacetylase complex has also been presented in HeLa cells [23], yeast [27] and chicken erythrocytes [30]. Recently, a mammalian histone deacetylase has been isolated by its strong affinity to trapoxin [35], a cyclic tetrapeptide inhibitory to all histone deacetylases analyzed so far [21,34]. Interestingly, that approach led to the isolation of just one histone deacetylase with a molecular weight of 55 kDa [35]. The isolated protein has a 60% sequence identity to Rpd3p, a well known yeast transcriptional regulator [46]. It is not known at present whether the high molecular weight histone deacetylase forms of yeast and chicken erythrocytes share sequence homology with *RPD3*, but the protein components of the high molecular weight HD2 complex of maize do not at all exhibit sequence

homology (Lusser and Brosch, unpublished results). Therefore, it could well be that HD1-A is a maize *RPD3* homolog, an assumption that will be tested by molecular analysis of the encoding gene.

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