Characterization of pea histone deacetylases

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Abstract

The present paper is the first report on histone deacetylases from plants. Three enzyme fractions with histone deacetylase activity (HD0, HD1 and HD2) have been partially purified from pea (*Pisum sativum*) embryonic axes. They deacetylate biologically acetylated chicken histones and, to a lesser extent, chemically acetylated histones, this being a criterion of their true histone deacetylase nature. The three enzymes are able to accept nucleosomes as substrates. HD1 is not inhibited by n-butyrate up to 50 mM, whereas HD0 and HD2 are only slightly inhibited, thereby establishing a clear difference to animal histone deacetylases. The three activities are inhibited by acetate, Cu^{2+} and Zn^{2+} ions and mercurials, but are only scarcely affected by polyamines, in strong contrast with yeast histone deacetylase. Several criteria have been used to obtain cumulative evidence that HD0, HD1 and HD2 actually are three distinct enzymes.

In vitro experiments with free histones show that HD0 deacetylates all four core histones, whereas HD1 and HD2 show a clear preference for H2A and H2B, the arginine-rich histones being deacetylated more slowly.

Introduction

Acetylation of ε-amino groups of lysyl residues is the most thoroughly studied histone post-synthetic modification (for a recent review, see[33]), although its precise roles are not yet fully understood. The turnover of acetyl groups depends upon the activity of two sets of enzymes catalyzing opposite reactions: histone acetyltransferases and histone deacetylases. It is obvious that a better characterization of these enzymes will help to understand the roles of histone acetylation.

Histone acetyltransferases have been described in animals (reviewed in [17]), yeast [16, 24] and plants [20, 22]. Histone deacetylases are less well known. They were first described and characterized almost 20 years ago [11, 12] and, since then, their presence has been reported in several animals [8, 14, 26] and

fungi [2, 30]. In some instances, a limited multiplicity of histone deacetylases has been detected. Chromatography on DEAE-cellulose columns yields two peaks of enzyme activity for calf thymus [13] and for a rat hepatoma tissue culture cell line [5]. To date, histone deacetylase has not been described in plants. Although Fujimoto [8] reported the presence in spinach leaves of an enzyme capable of removing acetyl groups introduced into histones by chemical acetylation with acetic anhydride, the enzyme was absolutely uneffective in deacetylating biologically labelled histones.

The present research is aimed at the study of histone deacetylases in plants. The knowledge of the properties of the enzyme(s), together with that of histone acetyltransferases [20, 22], will help to understand the role(s) of histone acetylation in plants and, eventually, it may result in a beter knowledge

of the similarities and differences between plants and other eukaryotes with respect to the structure and function of chromatin.

Materials and methods

Plant materials

Pea (*Pisum sativum* cv. Lincoln) were germinated as previously described [25]. Seedlings were raised after 60 h of germination and the embryonic axes were excised from the cotyledons and used for the subsequent experiments.

Extraction of histone deacetylases

Embryonic axes, in batches of about 70 g, were homogenized at 4 °C by grinding with acid-washed sand (axes/sand, 1:1, w/w) in 2 vol of buffer A (0.5 M NH_4Cl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 80 mM $NaHSO_3$, 15 mM Tris-HCl, pH 7.9). The homogenate was filtered through two layers of muslin and the retained material was washed twice with 0.5 vol of buffer A. The combined filtrates were stirred for 30 min in the cold and centrifuged at 27 000 g for 15 min. The supernatant was then centrifuged at 100 000 g for 1 h, the floating layer of lipids was removed and the supernatant was then dialyzed against buffer B (10 mM NH_4Cl, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 15 mM Tris-HCl, pH, 7.9) (two changes, 2 l each).

The dialysate was then loaded onto a column of DEAE-Sepharose CL-6B (17 cm×3 cm), previously equilibrated with buffer B. The column was then washed with 5 vol of buffer B. The retained proteins were then eluted with 1200 ml of a linear gradient of NH_4Cl, 10 to 350 mM, in buffer B, at a flow rate of 70 ml/h. Fractions showing enzymatic activity (see below) were pooled and concentrated by ultrafiltration in a Nucleopore cell fitted with an Amicon YM10 membrane, and operated under N_2 pressure.

Purification of histone deacetylases

Concentrated, pooled fractions containing histone deacetylase activity were further purified by gel permeation chromatography on a column (120 cm×1.8 cm) of Ultrogel AcA 34, equilibrated and eluted with a buffer similar to buffer B, except that it contained 35 mM NH_4Cl. Elution was carried out at a flow rate of 12 ml/h and 3.7 ml fractions were collected. Before assaying histone deacetylase activity, the fractions were made 200 mM in NH_4Cl by adding the appropriate amount of 3 M-containing buffer B.

Preparation of histone deacetylase substrates

Several substrates were used in this research. These include "biologically" acetylated chicken histones (either in nucleosomes or as free histones), and chemically acetylated histones and polyamines.

Chicken erythrocyte core histones were "biologically" acetylated by incubating a reticulocyte-enriched red cell preparation with [^{3}H]-acetate (5.0 Ci/mmol) as described by Ferenz and Nelson [7]. To obtain oligonucleosomes, nuclei were suspended at a density of 1.25×10^9 nuclei/ml in 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 and they were incubated at 37 °C for 30 min with 300 units/ml micrococcal nuclease. Reaction was stopped by adding 100 mM EDTA to a final concentration of 10 mM and chilling on ice. Digested nuclei were sedimented (1000 g, 10 min) and lysed in 0.25 mM EDTA. The lysate was then dialyzed against 0.25 mM EDTA (3 changes) and the resulting solution containing labelled oligonucleosomes was adjusted to $A_{260}=64$ and stored at -20 °C. Free histones were isolated following the procedure of Weintraub et al. [32]. Chicken histones obtained by this procedure were labelled to a specific activity of 2200 dpm/μg and the radiolabel was exclusively incorporated into core histones, as determined by electrophoresis and fluorography (see below).

Attempts to acetylate pea histones by incubating either isolated nuclei or chromatin with labelled acetyl-CoA or acetate were unsuccessful. Significant label was neither incorporated into histones after in-

cubating mesophyll protoplasts with [³H]-acetate under several conditions. Failure to prepare biologically labelled pea histones was surely related to the low level of pea histone acetylation and to the impossibility of specifically inhibiting deacetylase activity (see below).

Chemically acetylated histones were prepared by reaction with labelled acetic anhydride. Histones from either chicken erythrocyte or pea (5 mg) were dissolved in 400 μl of 50 mM sodium borate pH 9.0. Acetylation was started by adding 100 μl of a 0.26 μM solution of [³H]-acetic anhydride (1 mCi) in dioxane and the mixture was allowed to stand for 150 min at 0 °C. The solution was then made 0.25 M in HCl and the histones were precipitated with 8 vol of cold acetone. The precipitate was recovered by centrifugation, washed twice with acetone and dried under vacuum.

To acetylate spermine and putrescine, 10 mg of the polyamine were dissolved in 100 μl of the borate buffer and allowed to react overnight with 100 μl of the acetic anhydride solution at 0 °C. After adding 1 ml of water, the reaction mixture was loaded onto a Dowex-1 X4 column (6 cm×1 cm). The column was eluted with water and the fractions were tested for ninhydrine reaction. Both polyamines were recovered in the first 4 ml. The chemical and radiochemical purity of the preparations was checked by paper chromatography [6] and fluorography.

Histone deacetylase assay

To assay histone deacetylase activity along the purification procedure, 100 μl of the appropriate chromatographic fractions were incubated in 1.5 ml Eppendorf tubes with 10 μl of [³H]-acetate biologically labelled chicken histones (8 mg/ml). The mixture was incubated at 37 °C for 60 min and the reaction was stopped by adding 37 μl of 1.0 M HCl, 0.4 M acetic acid. Released labelled acetate was extracted with 700 μl of ethyl acetate [11] and 500 μl of the organic layer were mixed with 5 ml of 22-Normascint (Scharlau) and counted.

To study the pH dependence of the deacetylase activities, the enzymatic preparations, purified on Ultrogel AcA 34, were dialyzed against buffer B for the pH interval 7.1 to 9.1, or against 10 mM sodium phosphate buffer for pH 6.2 to 7.4. When applicable, the basic procedure for enzymatic assay mentioned above was modified by changing the appropriate parameter (temperature, NH₄Cl concentration, volume of labelled histone solution or pH). The influence of inhibitors was tested by including them in the assay mixture at the desired concentration. In some instances, namely when studying the dependence of the activity on the concentration of n-butyrate and other ionizable inhibitors, the assay mixture was supplemented with the appropriate amount of NH₄Cl in order to kept constant the ionic strength of the samples containing different amounts of the inhibitor.

The deacetylase activity towards polyamines was assayed in a similar fashion, by including a radio-labelled polyamine solution (final concentration 365 μg/ml) instead of the histone substrates.

Finally, in some instances, nucleosomes containing [³H]-acetylated histones were used as substrate for histone deacetylase assay. In this instance, the oligonucleosome preparation (see above) was thawed and enough solution was added to the incubation mixture to obtain a final concentration of 750 μg histone/ml. Therefore, the histone concentration in the assay is equivalent to that used with free histone assays.

Specificity of histone deacetylases

Electrophoresis and fluorography of labelled histones were carried out as described previously [16]. The amount of radiolabel present in each histone class was determined by integrating the intensity of the whole area of the corresponding band in the fluorogram in an IBAS-2000 image analyser system (Kontron). This integrated labelling density was normalized by dividing it by the integrated intensity of the Coomassie-stained band, determined as above from the stained gel. The resulting value, further referred to as specific labelling density (SLD), was taken as a measure of the specific activity of each [³H]-labelled histone class. Full details on this method will be given elsewhere.

Results

Multiplicity of histone deacetylase activities

The histone deacetylase activity in 60 h embryonic axes was resolved into three peaks by DEAE-Sepharose chromatography (Fig. 1). Peak 1 accounts for most of the apparent enzymatic activity recovered, but peaks 0 and 2 were consistently detected in all the experiments carried out with 60 h embryonic axes. The total enzymatic activity after DEAE-Sepharose chromatography was about 2.5 times higher than that found in crude extracts. This is probably due to the presence of inhibiting factors interfering with the enzyme activity. Earlier embryonic axes (after 24 h of germination) gave a pattern similar to that of Fig. 1 except that peak 0 was absent.

Fractions from the three peaks were pooled and they were further purified by gel permeation chromatography on Ultrogel AcA-34. Activity from peak 0 was recovered at an elution volume corresponding to $M_r \approx 90000$, whereas the activities from peaks 1 and 2 seemed to be associated with polypeptides in the range of $M_r \approx 100000$. Ultrogel chromatography typically resulted in a 3-fold increase of the enzyme activity per unit mass of protein.

The enzymatic extracts after Ultrogel chromatography were used to investigate the properties of histone deacetylases (HD). The activities present in peaks 0, 1 and 2 will be designated HD0, HD1 and HD2 respectively.

Histone deacetylases HD0, HD1 and HD2 differ in a number of properties. The optimum temperature for HD0 and HD2 is near 37 °C, whereas it lies in the range 27–32 °C for HD1. The ionic strength dependence of the three activities is also different (Fig. 2). HD1 has a narrow margin of optimum activity near 200 mM NH_4Cl, and HD2 shows a wider interval of optimum activity, but the activity of HD0 continuously decreases with increasing ionic strength. The pH dependence of enzyme activity also reflects some differences among the three deacetylase activities. For HD0 the activity is optimal at pH $\approx$ 7.7, whereas for HD1 and HD2 the optimum pH values are, respectively, 7.2 and 7.0. The latter two values are very similar, but the activity vs. pH profiles are quite different for both enzymes. The activity of HD1 sharply drops both at the alkaline and acidic sides of the optimum, but the inactivation of HD2 at alkaline pH values is smoother than in HD1 (not shown).
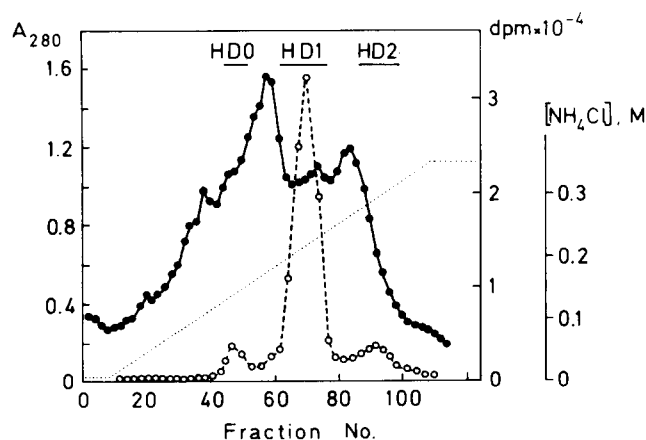

Fig. 1. Elution of pea histone deacetylases from DEAE-Sepharose CL-6B. A column of 3 cm×17 cm was loaded with centrifuged extract (see the text) from embryonic axes germinated for 60 h. Elution was carried out with 1200 ml of a linear gradient of NH_4Cl in buffer B, at a flow rate of 70 ml/h. Fractions of 12 ml were collected. ●, A_{280}; ○, histone deacetylase activity, expressed as dpm of released acetate under standard assay conditions; · · ·, NH_4Cl gradient. The fractions showing enzymatic activity were pooled as indicated by the horizontal bars.
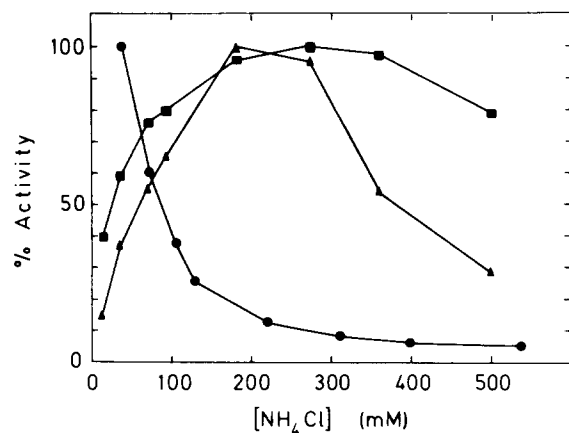
Fig. 2. Effects of ionic strength on the activity of pea histone deacetylases. ●, HD0; ▲, HD1; ■, HD2. Activity is expressed as percentages relative to the maximum activity achieved for each enzyme in this experiment.

Effect of inhibitors

Short-chain fatty acids, especially n-butyrate, are known as potent inhibitors of histone deacetylases from vertebrates [4, 21, 27], but histone deacetylase associated with yeast nuclei is not inhibited by n-butyrate nor propionate [2]. To test whether n-butyrate affected pea histone deacetylases, this potential inhibitor was included in enzymatic assays. As described under Materials and methods, in order

to avoid artifacts, the ionic strength was kept constant by adding the appropriate amounts of NH_4Cl. The results obtained (Fig. 3) show that n-butyrate has no effect on HD1 activity, and it causes only a slight inhibition on HD0 and HD2. At any rate, the effects of n-butyrate on pea histone deacetylases are not comparable to those on vertebrate enzymes. HD2, the most butyrate-sensitive of the pea deacetylases, retained about 60% of its activity in the presence of 50 mM n-butyrate, whereas this inhibitor, at a concentration of about 5 mM causes a remarkable supression of histone deacetylase activity (96−98%) in higher animals [5].

Acetate is, however, a potent inhibitor of pea histone deacetylases (Fig. 3). 50% inhibition is reached at about 2, 6 and 7.5 mM acetate for HD0, HD2 and HD1, respectively, and HD0 is 90% inhibited by 16 mM acetate. HD1 and HD2 are inhibited to a lesser extent and their behaviour is very similar. Preliminary results indicate that acetate is not a competitive inhibitor.

Some other potential inhibitors were tested, and the results are listed in Table 1. First, the effects of two protease inhibitors, PMSF and $NaHSO_3$ were tested to determine whether the deacetylase activity observed is, in some way, related to a proteolytic activity. $NaHSO_3$ does not inhibit HD1, and PMSF
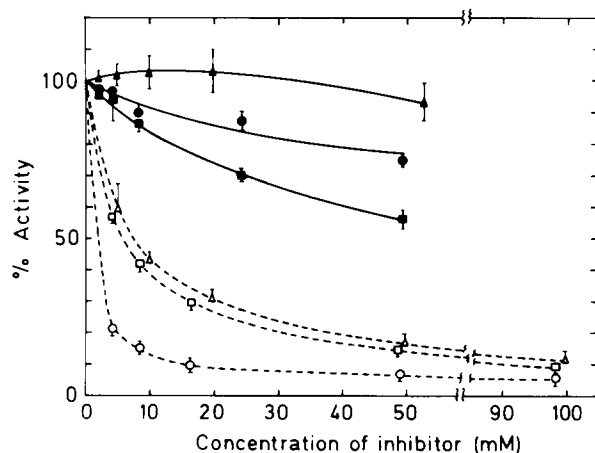

Fig. 3. Effects of potential inhibitors on the activity of pea histone deacetylases. ○, ●, HD0; △, ▲, HD1; □, ■, HD2. ●, ▲, ■, n-butyrate. ○, △, □, acetate.

Table 1. Effect of different potential inhibitors on the activity of pea histone deacetylases.

Inhibitor	Percent residual activity in		
	HD0	HD1	HD2
5 mM PMSF	15	20	17
5 mM NaHSO₃	42	100	71
5 mM ZnCl₂	0	0	0
5 mM CuCl₂	0	0	0
0.25 mM PMB	10	10	0
4.7 mM spermine	49	78	82
4 mM spermidine	nd	100	nd
5 mM choline	100	94	100
10 mM choline	nd	92	100
5 μM eserine	100	96	98

100% activity corresponds to the activity of each histone deacetylase in the standard assay (see Materials and methods), in the absence of added factors.
nd = not determined.

causes a remarkable inhibition of the three activities. However, the possibility that the release of the radiolabel were due to the hydrolysis of peptide rather that acetamide bonds can be ruled out. The assay conditions, which include an extraction step with ethyl acetate at acidic pH, would preclude the coextraction of positively charged peptides together with the unionized form of acetate. Further evidence against proteolysis will be provided by the experiments of Figs. 4 and 5. It can be seen that 5 mM $ZnCl_2$ or $CuCl_2$ completely repress the three activities and mercurials, such as PMB, also have a clear effect. A similar result was found in the early studies of Inoue and Fujimoto [12], working with the calf thymus enzyme. This suggest that the enzymes possess essential thiol groups. Spermine is only a weak inhibitor of pea histone deacetylases, if compared with the effect of the polyamine on the yeast enzyme [28]. The activity of the latter is completely abolished at about 2 mM spermine, whereas pea HD0 is only 50% inhibited by 4.7 mM polyamine, its effect on HD1 and HD2 being still less pronounced. Our finding with spermidine is even more remarkable, as this polyamine, which is a potent inhibitor of histone deacetylases from yeast [28] and diminishes as well the activity of deacetylases from higher organisms [15], has no effect on pea HD1.

We also tested the effects of choline and eserine on pea histone deacetylases, because it has been suggested that acetylcholinesterase may possess some histone deacetylase activity [1]. As shown by the negative results in Table 1, this is probably not the case for pea HD enzymes.

The question of whether non-histone HMG (high mobility group) proteins alter the activity of histone deacetylases is rather controversial. Reeves and Candido [19] suggested that HMG1 and HMG2 activate and HMG14 and HMG17 inhibit histone deacetylase in vitro, but Mezquita et al. [18], in a different system, found that HMG17 does not inhibit histone deacetylase, which was stimulated by ubiquitin. To test whether these proteins had any effect on the pea enzymes, chicken HMG1/2 and HMG14, prepared according to Walker and Johns [29], were added to the deacetylase assay mixture. Neither HMG1/2 nor HMG14 (200 μg/ml) had any effect on HD0 and HD2. HD1 activity was only slightly enhanced

($<10\%$) by HMG14 (200 μg/ml). HMG1, below 100 μg/ml, did not affect HD1 activity, but at 200 μg/ml causes a 50% activation. Ubiquitin (200 μg/ml) had no effect on HD0 and HD2 activities, and HD1 was activated only to a small extent ($\approx 10\%$).

Histone deacetylation in nucleosomes

The three pea histone deacetylases are able to deacetylate histones when using nucleosomes as substrate. Chicken nucleosomes, containing labelled histones, were prepared by incubation of nuclei with [^{3}H]-acetate and used in the enzymatic assay as described under Materials and methods. The results of a typical experiment are given in Table 2. The three enzymatic preparations are able to accept oligonucleosomes as substrate, although they prefer the free forms of the histones. This preference for free histones is more marked in HD0 and HD2.

Substrate specificity of pea histone deacetylases

In addition to "biologically" acetylated chicken histones, either free or incorporated into oligonucleo-

Table 2. Nucleosome deacetylating activity of pea histone deacetylases.

Substrate	Deacetylase activity with		
	HD0	HD1	HD2
Chicken free histones (biologically labelled)	14776 ± 224	8171 ± 250	2380 ± 165
Chicken oligonucleosomes	2057 ± 53	3839 ± 207	400 ± 108
(Activity toward oligonucleosomes/activity toward free histones) $\times 100$	14	46	17

Activities are given as dpm of released acetate in the presence of 40 mM NH_4Cl. In every case, the activity of input histones (either free or in nucleosomes, see Materials and methods) was 90000 dpm.

Table 3. Activity of pea histone deacetylases towards different acetylated substrates.

Substrate	Deacetylase activity with		
	HD0	HD1	HD2
Chicken erythrocyte free histones, biologically labelled	100	100	100
Chicken erythrocyte free histones, chemically labelled	69	44	22
Pea free histones, chemically labelled	30	15	6
Acetylspermine	0	1	0
Acetylputrescine	0	0	0

As the specific activity of each substrate was different, released acetate was first evaluated as percentages relative to the input dpm in the assay, and these figures were then recalculated, for each of the three enzymatic activities relative to 100 for biologically acetylated chicken histones.

somes, some other acetylated compounds were tested as substrates. The results obtained are listed in Table 3. It is noteworthy that chemically acetylated histones, obtained by treating the protein with labelled acetic anhydride (see Materials and methods) are poorer substrates than biologically acetylated histones. This is true even when using homologous, pea histones. There is an obvious ex-

planation for this result, which was previously suggested by Inoue and Fujimoto [11] in their pioneering work on calf thymus histone deacetylase: whereas acetyltransferase-catalyzed *in vivo* acetylation of histones results in the modification of ϵ-NH_2 groups of specific lysyl residues for each core histone class [33], chemical acetylation with acetic anhydride may result in a more extensive modification, even afecting H1 and the N-terminal groups of H2B and H3. These *extra* acetylated groups would not be recognized by histone deacetylases. In the light of this interpretation, our preparations may be considered as true histone deacetylases, capable of discriminating between biologically and chemically acetylated histones. Finally, Table 3 shows that acetyl polyamines are not deacetylated at all.

At the present time, the available data indicate that, at least H4 [20] and H3 [2] are acetylated in pea and all four core histones are acetylated in higher animals. To determine whether our HD preparations react differentially with the acetylated four core histones, we performed a series of experiments, one of which is shown in Fig. 4. It is obvious from this figure, that incubation of [^{3}H]-acetylated histones with HD2 resulted in a preferential cleavage of acetyl groups from H2A and H2B. In addition, these results clearly show that the removal of the radio-
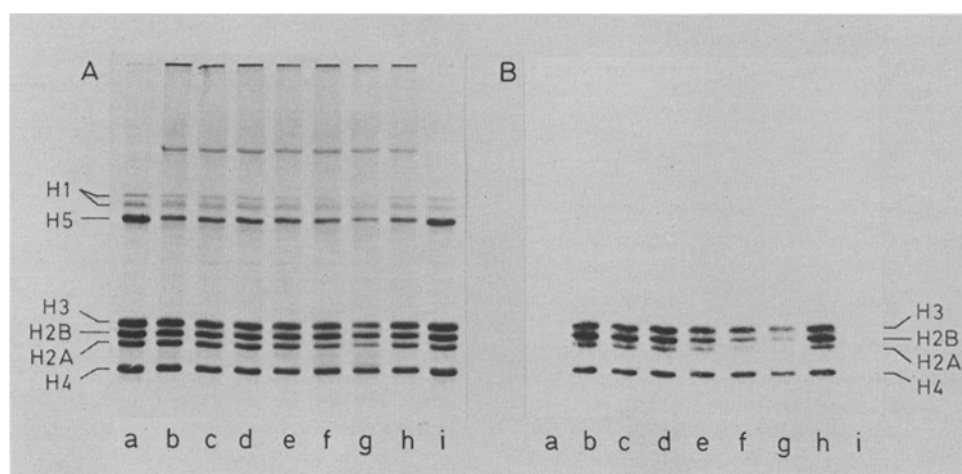

Fig. 4. Specificity of histone deacetylation by HD2. Chicken histones, biologically labelled with [^{3}H]-acetate, were incubated with a purified preparation of HD2. Aliquots from the reaction mixture were taken at several times, the histones recovered and electrophoresed. Panel A shows the Coomassie-stained gel and panel B the corresponding fluorogram. In lanes a and i unlabelled chicken histones were run. The aliquots were taken at: b, 0; c, 10; d, 20; e, 40; f, 80 and g, 160 min. The progress of enzymatic deacetylation is easily seen by the disappearance of labelled acetate (panel B), especially in H2A and H2B. Lane h shows the result of incubating the histones for 160 min with an enzymatic extract boiled to inactivate histone deacetylase activity. No removal of acetate was apparent under these circumstances.

label is not related to proteolysis of histones, as revealed by the absence of degraded histone material, both in the Coomassie-stained gel and in the fluorogram. In order to quantitate the time-course of acetyl hydrolysis, we followed the procedure described under Materials and methods. Figure 5 shows the time-course of acetyl cleavage in relative SLD values (see Materials and methods), for each of the four core histones, in incubations with HD0 and HD1 (electrophoreses not shown) and HD2 (gel of Fig. 4). Each of the pea histone deacetylases shows a different histone specificity. HD0 deacetylates all four core histones, with a slight preference for H3,

but HD1 and HD2 preferentially remove acetate from H2A and H2B, the arginine-rich histones, H3 and H4, being deacetylated more slowly. There is also some difference between HD1 and HD2: while the former removes acetate from H2B more efficiently than from H2A, the latter enzyme preferentially acts on acetylated H2A.

Discussion

In the present paper we describe the partial purification and characterization of three histone deacetylases from pea embryonic axes. Low molecular weight acetylated substrates are not hydrolyzed by any of the three activities (Table 3) and chemically acetylated histones are poorer substrates than biologically acetylated histones. These are the most important arguments supporting the view that we are dealing with true histone deacetylases. Table 4 summarizes the evidence supporting that the three activities, HD0, HD1 and HD2, represent three separate molecular species, rather than artifactual aggregates of a single protein.
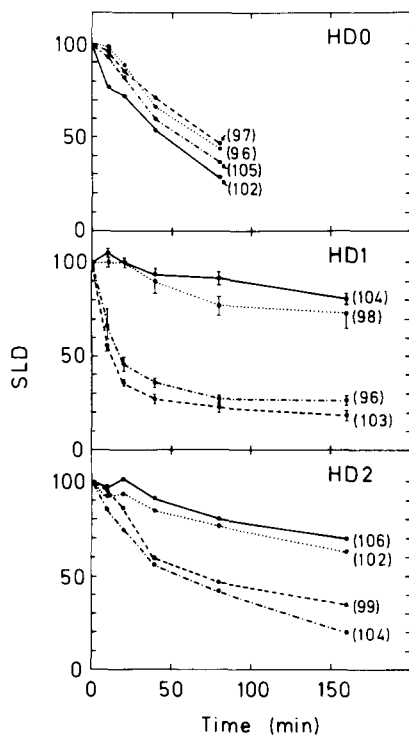

Fig. 5. Normalized specific labelling density (SLD) plotted versus time for the three histone deacetylases. From the experiment of Fig. 4, and parallel experiments conducted with HD0 and HD1, the intensity of the label in the fluorogram was integrated by an image analyser and divided by the integrated intensity of the corresponding Coomassie-stained band. The resulting values were normalized to 100 and plotted. In the middle panel an example of the standard deviations obtained with this procedure is given. The figures in parentheses represent the specific densities of samples incubated during the total time of the experiment with boiled enzymatic extracts (analogous to the sample of lane h in Fig. 4. (————), H3; (— — —), H2B; (— · —), H2A; (· · ·), H4.

Table 4. The multiplicity of pea histone deacetylases.

Criterion of distinction	Enzymatic activity with distinct or unique properties		
T-dependence of enz. activity		*HD1*	
Ionic strength-dependence	**HD0**		
pH-dependence	*HD0*		
Effects of n-butyrate		*HD1*	
Effects of acetate	*HD0*		
Effects of bisulphite		**HD1**	
Effects of HMG proteins		**HD1**	
Activity vs. oligonucleosomes/ activity vs. free histones		*HD1*	
Chemically acetylated histone as substrate	*HD0*	*HD1*	*HD2*
Histone class specificity	**HD0**		

The name of an enzymatic activity in italics means that, by using the corresponding criterion, enough quantitative differences between the indicated enzyme and the two other forms were found (see text) to consider the former as a distinct enzyme. A name in bold type means that the enzyme exhibited unique properties, i.e., it showed a qualitatively different behaviour with respect to the other forms, and not only a quantitative difference.

Pea histone deacetylases resemble the *Physarum* enzyme in that they are only slightly inhibited by butyrate [31]. Plant and slime mold enzymes range in this respect between vertebrate and yeast enzymes. Arfmann and Haase [3] found a clear increase of the apparent acetylated forms of H4 when *Agrobacterium*-transformed and untransformed cell lines of *Nicotiana tabacum* were grown in the presence of 5 mM butyrate. We do not know whether the very slight inhibition *in vitro* caused by 5 mM butyrate on HD0 and HD2 could be responsible for the accumulation of acetylated histones. Moreover, the situation *in vivo* may be quite different from our *in vitro* conditions. To explain the insensitivity of the yeast enzyme to butyrate *in vitro*, Alonso and Nelson [2] proposed that this non-competitive [5] inhibitor may interact with a separate regulatory subunit of the enzyme, which could either be lost or damaged during the isolation procedure. This explanation may also be valid for our pea enzyme preparations.

Acetate, which do not affect the yeast enzyme and only slightly inhibits vertebrate deacetylases [5, 21], exhibits a noticeable inhibitory effect on pea deacetylases. The pea enzymes also differs from yeast histone deacetylase in that they are not inhibited by polyamine, whereas 2 mM spermine or 5 mM spermidine fully inhibit the yeast enzyme [28].

The influence of chicken HMG proteins on the activity of pea histone deacetylases is very limited. The only effect we found was a slight activation of HD1 by 200 μg/ml HMG1/2. Since plant and animal HMG proteins are different [23], it would be interesting to study the effect of the homologous HMG proteins. There is, however, some immunological cross reactivity between plant HMGd and chicken HMG1/2 (but not HMG14; see [23]), suggesting that the activation of pea HD1 by HMG1/2 may be specific.

The experiments shown in Figs. 4 and 5 prove that HD0 has a different histone specificity than HD1 and HD2. Of course, the specificity pattern may change when homologous nucleosomes are used as substrates *in vivo*, but our results from *in vitro* experiments are a consequence of the existence of heterogeneity in histone deacetylases. Hay and Candido [9, 10] have described the existence of a complex form of histone deacetylase in HeLa cells, in which the enzyme is associated with some nuclear matrix proteins and other non-histone proteins, in such a way that the complex is stable even in 1–2 M NaCl. Free histones are a poor substrate for this complex form of the deacetylase. It is not known whether the deacetylase complex represents a widespread mode of occurrence of the enzyme in eukaryotes and the soluble forms capable of acting toward free histones, as those reported here, are artifacts produced during isolation. The results of Alonso and Nelson [2] suggest for the yeast enzyme the existence of a soluble native form, so that the possibility exists that some organisms possess enzymatic complexes, whereas other eukaryotes contain soluble forms of the enzyme or even share both forms of deacetylases. We are now trying to develop an assay procedure to answer this question in plants.

Acknowledgements

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