PURIFICATION AND PROPERTIES OF A HISTONE-SPECIFIC PROTEASE FROM RAT LIVER CHROMATIN

Effect on acylated histones

Giampietro RAMPONI, Paolo NASSI, Gianfranco LIGURI and Gianni CAPPUGI
Istituto di Chimica biologica, Università di Firenze, Italy

and

Santiago GRISOLIA
Instituto de Investigaciones Citologicas, Amadeo de Saboya, 4, Valencia – 10, Spain

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1. Introduction

Chromatin contains proteases which act on histones [1–7]. It has been suggested that such proteases play a role in gene regulation. Histones, like other proteins, can be covalently modified by such reagents as the highly reactive acyl phosphates. Of particular interest is the acetylation of epsilon amino groups of lysine residues within the polypeptide chain of histones, which has been correlated with gene activation, while N-terminal acetylation has been associated with histone synthesis [8]. Indeed, it should be noted that an ϵ-acetyl-lysine deacylase presumably functions 'in vivo' to clear ϵ acyl proteins and that chemotrophic modifications may be of significance in a number of physiological phenomena, including protein turnover [10].

We present here a simple procedure for obtaining a purified histone protease in excellent yield from rat liver chromatin, together with some of its properties, especially the opposite effects of carbamylation and acetylation on the relative susceptibility of histones to degradation by the purified protease.

2. Material and methods

Male Wistar rats, 200–250 g, purchased from S. Morini, S. Polo d'Enza, Italy, were used.

Lysine rich histones (type III S), hemoglobin and casein were purchased from Sigma Chemical Co., St Louis, MO. Bovine serum albumin was from Merck, myoglobin and soybean trypsin inhibitor from Koch-Light Laboratories, lysozyme and β-fructosidase were from Boehringer and Soehne GmbH. A whole histone preparation was obtained by the procedure in [11]. Acyl phosphatase was purified from horse muscle [12]. Homocitrulline was synthesized [13]. Chromatin was isolated from rat liver homogenates according to [14], except that fresh liver was used. Sepharose 6B, Sephadex G-200 and Sephadex G-25 were from Pharmacia Fine Chemicals; acrylamide from Eastman Organic Chemicals; *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine from Serva; sodium dodecylsulphate from BDH Chemical Ltd. All other reagents were of the highest purity available.

Protease was assayed essentially as in [7], as follows: routinely the assay mixture contained in 0.2 ml: NaCl 40 μmol, Tris (pH 8) 2 μmol, histones or other protein substrate 0.2 mg and enzyme (0.01–1.0 μg protein). After incubation in screw-capped test tubes for 8 h at 37°C, 0.5 ml ninhydrin reagent (0.4 g ninhydrin, 80 ml 95% ethanol, 1 g $CdCl_2$, 10 ml acetic acid and 20 ml water) were added. The tubes were heated for 4 min at 100°C, cooled and the A_{506} was measured in a Beckman model DB spectrophotometer. One unit of protease is defined as that which causes an increase

in absorbance of 0.01 using 10 mm light path cells. Specific activity is defined as units/mg protein estimated by the method in [15] or by ultraviolet absorption.

The purified enzyme was analyzed by sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis using Tris–glycine buffer [16], 10% gel (containing 1.0% SDS) and 3 mA/tube for 3 h at room temperature. The gels were stained with 0.5% amidoblack in methanol–acetic acid–water (50:10:50, v/v/v) and destained with the same solvent.

The molecular weight of the enzyme was determined by SDS–polyacrylamide gel electrophoresis [17], using a 15% gel and 0.1 M phosphate buffer, pH 7.2, containing 1% SDS and 8 mA/tube for 7 h at room temperature. Staining and destaining were as described above. The gels were scanned with a Gilford 2400 spectrophotometer equipped with 2410 linear transport attachment. The relative mobility of each protein was related to that of bovine serum albumin.

Lysine-rich histones were carbamylated and acetylated as follows: 100 mg histone were taken into 10 ml 150 mM KCNO, pH 8.5. After 36 h the pH was brought to 2 with 3 N HCl and 200 ml acetone were added. The precipitate was washed repeatedly with ether, dried and then dissolved in 2 ml 10 mM HCl. It was then applied to a column (1 × 70 cm) of Sephadex G-25 and eluted with 10 mM HCl at a 28 ml/h flow rate. The content of homocitrulline in the modified histone was measured [18]. For the acetylation, the procedure in [19] was employed, with some modification as follows: 100 mg histone were dissolved in 2.5 ml 2 M sodium acetate, pH 8.1, 200 μmol acetic anhydride were added and left to react for 44 h at 0°C; 5 ml absolute ethanol were then added and the precipitate was washed several times with this solvent. About 20% of the acetyl groups in the preparation were hydroxylamine labile. The acetyllysine was estimated from the lysine content of acid and enzymatic hydrolysates and by direct identification of acetyllysine [20].

Native, carbamylated and acetylated histones were incubated with 0.1 μg purified protease under the standard conditions described above, except that the incubation was for 10 min. The incubation mixtures were cooled in an ice-bath and then lyophilyzed. The lyophilyzed material was dissolved in 20 μl H_2O, dialyzed against the same solvent, and subjected to polyacrylamide disc electrophoresis according to [16]. The gels were stained and scanned as described above.

3. Results

3.1. *Purification of the enzyme*

All operations were carried out at 0–4°C. To purified chromatin from 100 g rat liver (10 A_{260}/ml in 10 mM Tris, pH 8) crystalline NaCl was added, with stirring, to 0.7 M. After further stirring for 48 h, it was centrifuged at 50 000 × *g* for 1 h. The precipitate was discarded. The supernatant was concentrated by ultrafiltration (Amicon UM-2 membrane) and then applied to a Sepharose 6B column (100 × 3 cm) equilibrated and eluted with 10 mM Tris, pH 8, containing 0.7 M NaCl. Figure 1 shows the elution diagram. Active fractions were pooled and diluted with approximately the same volume of Tris buffer (to 0.4 M NaCl). After concentration to 20 ml by ultrafiltration, the enzyme preparation was introduced (in 4 portions) in a Sephadex G-200 column (120 × 4 cm), equilibrated and eluted with 10 mM Tris, pH 8, containing 0.4 M NaCl. Figure 2 depicts a typical elution pattern. The pooled fractions had spec. act. 83 000, or over an 800-fold purification, with respect to the starting chromatin preparation. The steps of the purification procedure are summarized in table 1.

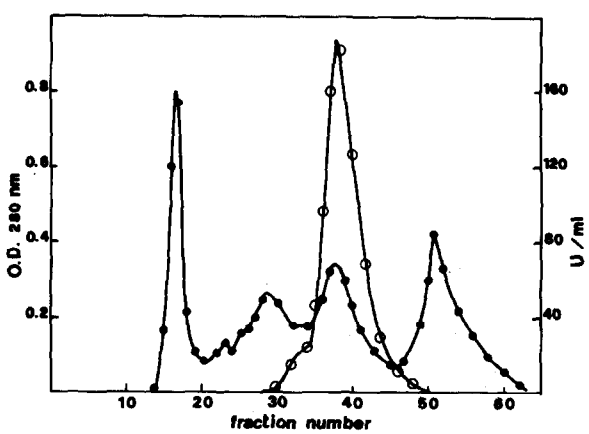

Fig.1. Elution diagram of the enzyme from a 100 × 3 cm column of Sepharose 6B. (•——•) A_{280} nm; (○——○) protease activity.

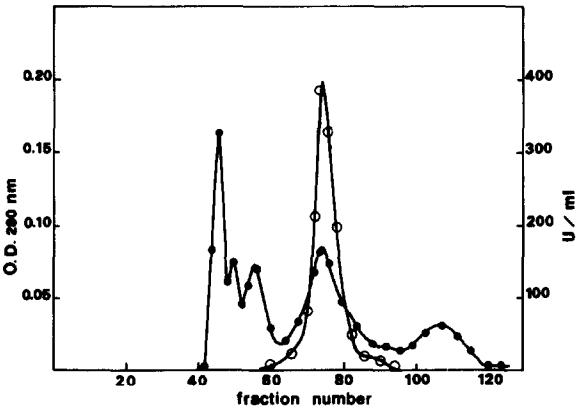

Fig.2. Elution profile of the enzyme from a 120 × 4 cm column of Sephadex G-200. (•——•) A_{280} nm; (○——○) protease activity.

Figure 3 shows the SDS disc electrophoresis pattern of the protease at the last step of purification. A sharp band was obtained (comprising over 95% of the protein applied to the gel).

3.2. *Properties of the purified enzyme*

A molecular weight of 103 000 was calculated for the purified enzyme by SDS disc electrophoresis. β-Fructosidase, bovine serum albumin, ovalbumin, myoglobin and muscle acyl phosphatase were run as standards.

The purified protease has a high specificity for lysine-rich histone, as expected [21]. Considering whole histone as 100%, values for other substrates tested were: lysine rich histone 160, hemoglobin 21, myoglobin 9, lysozyme 4.5, casein 0.1 and bovine serum albumin 0.

The purified protease, assayed with lysine-rich histone, shows a pH optimum with Tris buffer of 7.65 (fig.4). It was stable for at least 2 months at 0°C.

The protease is completely inhibited by 10 mM EDTA and 1 mM Cu^{2+}, Fe^{2+} and Zn^{2+}, while 1 mM Mg^{2+} and Mn^{2+} were activating (126% and 166%, respectively). With other substances tested the following % activities were obtained: 0.1% SDS, 12; 1 mM EDTA, 46; 50 mM HSO_3^-, 47; and 0.5 mg/ml soybean trypsin inhibitor, 83.

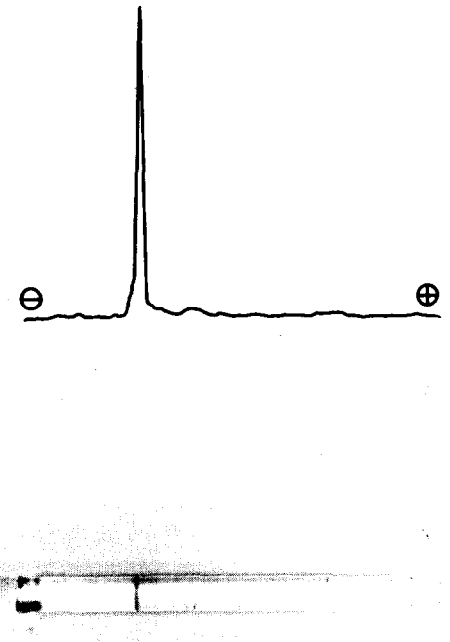

Fig.3. Electrophoresis pattern and densitometry of the enzyme at the last step of purification.

Table 1
Enzyme purification

Step	Vol. (ml)	Protein (mg)	Activity (units)	Spec. act. (units/mg protein)	Yield (%)
Purified chromatin	200	900	120 000	103	100
Salting with NaCl	30	240	93 000	391	78
Sepharose 6B	20	42	56 300	1340	47
Sephadex G-200	5	0.5	41 500	83 000	34

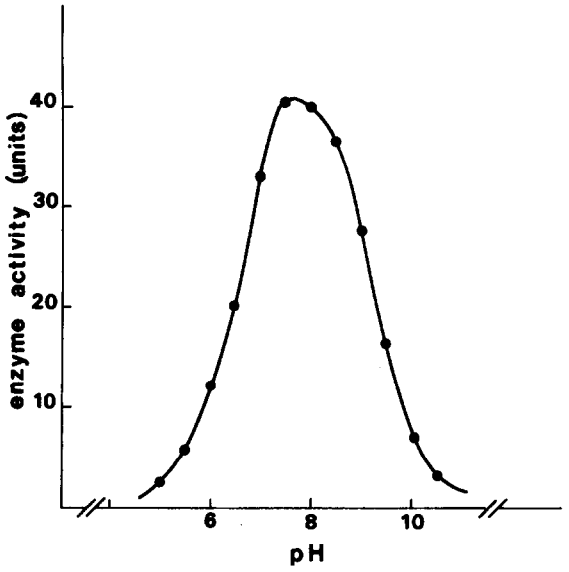

Fig.4. pH optimum of purified protease.

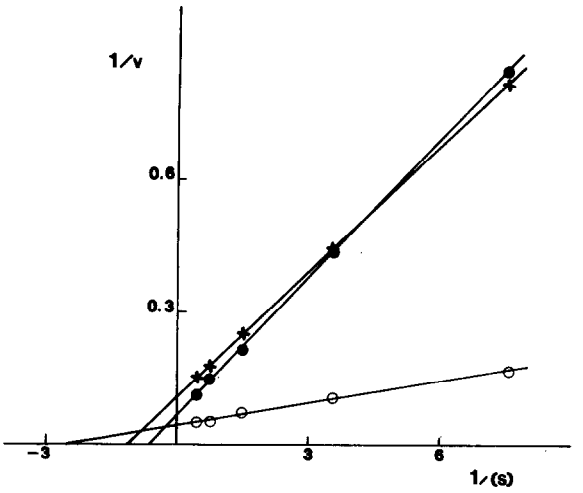

Fig.5. Lineweaver-Burk plots of purified protease towards native (•——•), carbamylated (X——X) and acetylated (○——○) lysine-rich histones. Substrate concentration is expressed as mg/ml, velocity as units, defined as reported in the text.

3.3. *Activity towards modified histones*

As reported in table 2, carbamylation of lysine-rich histone (35% total lysine residues) results in a significantly decreased degradation of this protein by the purified protease. On the other hand, acetylation (24% lysine residues transformed into acetyl-lysine) enhances the susceptibility of lysine-rich histone to proteolytic degradation. Both chemotrophic modifications are associated also with changes in affinity. In fact, as indicated by Lineweaver-Burk plots (fig.5), carbamylation and particularly acetylation of lysine-rich histone, enhances markedly the affinity of the protease, giving semi-maximal velocity at 0.93 mg/ml and 0.36 mg/ml respectively, while semi-maximal velocity with the unmodified histone was obtained at 1.54 mg/ml. The marked changes in the susceptibility to proteolytic attack induced by acetylation and carbamylation of histones are indicated also by electrophoretic analysis.

Table 2
Activity of purified proteinase towards native and modified lysine-rich histones

Lysine-rich histones	Activity (units)
Native	75 ± 2
Carbamylated	45 ± 2
Acetylated	120 ± 5

4. Discussion

The properties of the highly purified protease described here are similar to those of the enzyme purified in [7]. However, the purification procedure presented here results in a much higher yield.

The enzyme described here and the protease isolated from rat liver mitochondria [22] have a similar substrate specificity, however, they show marked differences in molecular weight and in effect of monovalent and divalent ions. Also, EDTA had no effect on the mitochondrial enzyme and soybean trypsin inhibitor, a strong inhibitor of the mitochondrial enzyme, was almost completely ineffective on the protease described in the present paper.

It is of much interest that modified proteins, particularly those in which amino acid analogues have replaced several amino acids, turn over or are attacked faster; indeed, glutamate dehydrogenase, an enzyme

extraordinarily susceptible to carbamylation but remaining antigenically indistinguishable from the native enzyme [23], is much more susceptible to pronase, trypsin and pepsin when carbamylated, in contrast to histones. Thus, while high susceptibility of histones to the purified enzyme is not compatible with the low turnover rate of these proteins in cell nuclei, the lower hydrolysis rate following carbamylation and the higher rate following acetylation suggest that chemotrophic modifications of histones could serve to regulate protease activity and, consequently, the turnover of nuclear proteins. However, whether the role of the protease described here is related to degradation of histones 'per se' and/or contributory to the release of these proteins from DNA during gene derepression, or that the altered susceptibility resulting from acetylation may serve as a model to be developed further, remains to be clarified.

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