A HISTONE PROTEASE OF RAT LIVER CHROMATIN

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Summary: Normal rat liver chromatin contains a protease activity with a marked preference for histone or deoxyribonucleohistone as substrate. This histone protease has been purified 20 to 30 fold from chromatin and has been analyzed for salt and pH dependence as well as for substrate specificity. The isolated enzyme appears to be similar to that responsible for the endogenous degradation of histones in calf thymus chromatin as reported by Bartley and Chalkley (1); however, its presence in adult rat liver, a tissue with very low cell turnover (and therefore low histone turnover), argues against the enzyme having only an autolytic function and suggests that it may play a role in gene derepression, etc.

It has been observed previously that isolated chromatin contains protease activity capable of degrading the histones present (2, 3, 4, 5). Since the majority of the evidence available to date suggests that the association of histone with DNA prevents gene transcription (6, 7), an enzyme which could remove histone from DNA by specific proteolysis might play a role in gene derepression. [It is to be noted that the bulk of the histone in a cell is extremely stable (8); special conditions altering the state of the histone to make it susceptible to the protease would have to be invoked by such a model (e.g., histone phosphorylation).] We have partially purified and characterized a histone protease of rat liver. Although the protease may play only an autolytic role (1), the low cell turnover in liver and the lack of any alternative mechanisms for the dissociation of histones [other than histone I (9, 10, 11)] from DNA *in vivo* make the above model attractive.

METHODS

Enzyme purification. Chromatin was prepared from frozen rat liver (Pel-Freeze Biologicals, Inc.) as described by Elgin and Bonner (12). All operations were carried out at 0-4°C. The purified chromatin was diluted to a final concentration of 8-10 OD_{260}/ml and brought to 0.7 M NaCl by dropwise addition of 2 M NaCl-0.01 M Tris, pH 8. After stirring slowly overnight, the chromatin was centrifuged for 4 hr at ca. 100,000 g. The supernatant was collected and passed rapidly through a 2.3 x 80 cm column of Sepharose 6-B to separate the remaining DNA from the extracted protein. Following concentration of the protein by ultrafiltration (Amicon PM 10 membrane), a second pass through the same column allowed the protease peak to be separated from most of the protein (Figure 1).

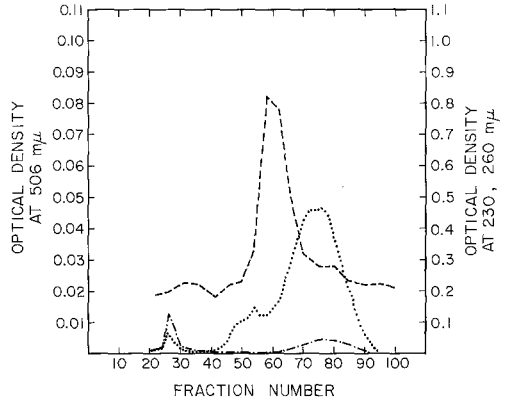

Figure 1. Purification of rat liver histone protease on Sepharose 6B. Second pass. ---- enzyme activity (ninhydrin); •••• OD_{230}; •-•-• OD_{260}. Fractions 54-66 pooled for use.

Enzyme assay. For purposes of purification and characterization of the protease a quantitative ninhydrin assay for production of N-terminals and/or gel electrophoresis of the substrate proteins were employed. In the standard enzyme assay, protease preparations of ca. 0.1 mg/ml protein were mixed with an equal volume of substrate at 1 mg/ml and incubated at 37°C, 0.005 M Tris, pH 8, 0.35 M NaCl for 4 hr. The reaction was stopped by putting the incubation tubes on ice and adding either an excess of electrophoresis

medium or 0.5 ml of ninhydrin reagent to 0.2 ml of the sample. [Ninhydrin reagent is 0.4 gm ninhydrin, 80 ml 95% ethanol, 1 g $CdCl_2$, 10 ml acetic acid, and 20 ml water (13)]. The ninhydrin assay tubes were capped, put in a boiling water bath for 4 min, cooled quickly and the sample measured for absorption at 506 mμ. In this assay absorption is linear with concentration for all amino acids and proteins tested, although extinction coefficients for the assay with different amino acids can vary by a factor of two or less (14). Polyacrylamide gel electrophoresis of the substrate protein, either in SDS (12) or urea (15) systems, proved to be a more sensitive assay for the cleavage of histones into large fragments.

Substrates. Rat liver histones for routine assays were prepared from purified chromatin (15). Unless noted, other proteins were purchased from Sigma or Calbiochem. Protein concentrations were determined by the method of Lowry et al. (16) using bovine serum albumin as a standard except that histone substrates were routinely standardized by the ultraviolet absorption at 230 mμ using $\epsilon = 4.15$ (l/cm-g) (12).

RESULTS

Characteristics of histone protease. A reproducible peak of protease activity elutes from Sepharose 6-B just ahead of the major protein peak. It represents a 20 to 30 fold purification of the enzyme from chromatin. Protease at all stages of purification is relatively stable and has been stored on ice for several weeks without loss of activity. Activity is partially destroyed by heating to 60°C for 10 min and totally destroyed by 100°C. The enzyme is completely inhibited by 0.05 M sodium bisulfite. The pH and salt dependence of protease using free histone substrate were determined by ninhydrin assay (Figure 2). Degradation is greatly inhibited by pH below 7.5 and has a maximum at about pH 8.2. Assays of endogenous activity in purified chromatin show substantially reduced degradation of histone at pH 7 and below (unpublished data). A protease activity present in acid-extracted rat liver histones is also inhibited below pH 7.5, as

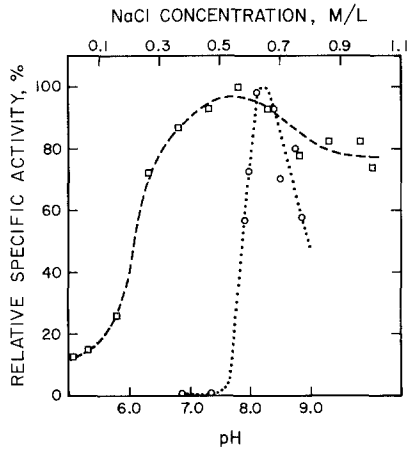

Figure 2. Effect of salt ---- and pH ···· on enzyme activity.

well as by 0.05 M sodium bisulfite. High NaCl concentrations do not inhibit degradation; the reduced activity at low salt may be due to aggregation.

Specificity of purified protease on histones in chromatin. Highly concentrated purified protease was added to freshly made chromatin and incubated at pH 6.7 and pH 8.0. Analysis of histone degradation by urea gel electrophoresis shows that purified protease has the same specificity for histones in chromatin as endogenous protease and generates the same breakdown products (Figure 3). Histone fraction I disappears first followed by II and IV; the effect on histone III may be obscured by the presence of a breakdown product. The lower pH reduces, but does not abolish, protease activity.

Substrate specificity. Table I summarizes the results obtained using the ninhydrin assay. Both SDS and urea polyacrylamide gels verify the relative stability of protamines under conditions in which histones are rapidly degraded. Hemoglobin was denatured in 8 M urea-0.125 M NaOH for 1 hr, neutralized, and assayed in 3 M urea. Under these conditions histones are attacked by protease. Although no ninhydrin response could be detected,

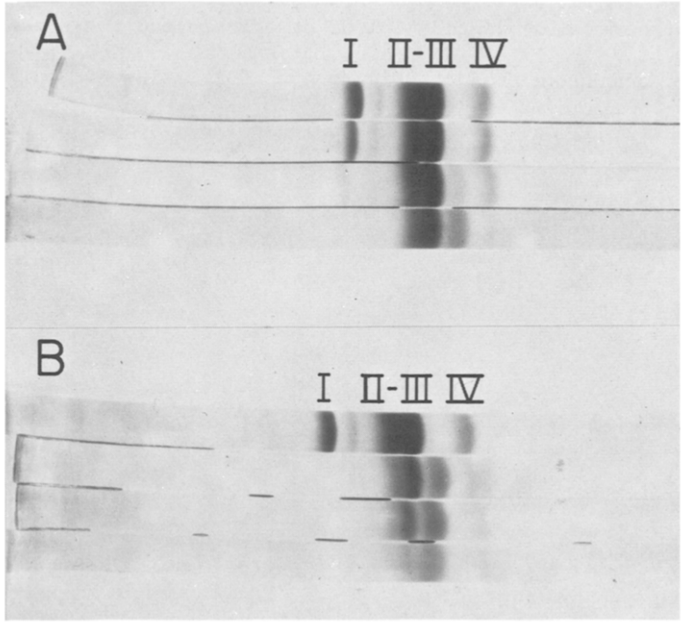

Figure 3. Degradation of histones of chromatin (A) without and (B) with additional histone protease. Incubation at 37°C, 0.005 M Tris, pH 8, 0.04 N NaCl, chromatin concentration 10-12 OD_{260}/ml for 0, 2, 4 and 8 hours in each case. Urea gels (run left to right) of the histones extracted with 0.4 N H_2SO_4 following incubation (15).

TABLE I

Substrate	Concentration (nM)	Relative Activity per nM Substrate
histone	0.07	100%
protamine (purified on Sephadex G-25)	0.10	9%
casein	0.06	18%
hemoglobin (denatured in alkaline urea)	0.08	0%
RNase	0.10	5%
lysozyme	0.09	2%
polylysine HBr (Miles Yeda)	0.02	320%

SDS gels show a slow degradation of hemoglobin and the appearance of a prominent breakdown product (Figure 4). Polylysine and polyalanine are good substrates, but casein, ribonuclease and lysozyme are not.

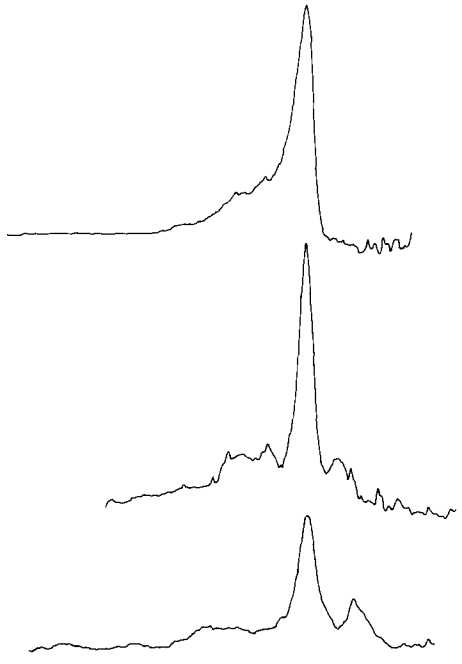

Figure 4. Degradation of hemoglobin by histone protease. SDS gels run left to right. Incubation for 0, 4 and 8 hours.

DISCUSSION

The data at hand indicate that the chromosomal protease preferentially degrades histones. Whether this selectivity is the consequence of the relatively open structure of histones or the concentration of particular peptide bonds (such as lys-lys) has not yet been determined. Marushige and Dixon (11) have recently reported evidence suggesting that phosphorylated histones are removed from DNA by such a histone-specific protease during trout spermatogenesis. The presence of histone protease in rat liver suggests that the enzyme may play a general role in gene derepression.

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