LYSOSOMAL ORIGIN OF CHICKEN ERYTHROCYTE NUCLEAR PROTEASE

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1. Introduction

Proteolytic degradation is encountered during the purification of histones [1] and combatted by the inclusion of the inhibitors bisulphite [2] or phenylmethylsulfonyl fluoride (PMSF) [3] in the isolation buffers. It is generally assumed that the proteolytic activity arises from chromatin-bound proteases [4] rather than from contamination of nuclei preparations with proteases originating from other organelles [5]. Neutral proteases bound to chromatin have been described for calf thymus [6] and rat liver [7,8]. These enzymes have been shown to be serine proteases on the basis of diisopropylfluorophosphate binding [6,7]. In addition, regenerating rat liver nuclei have been shown to contain a thiol protease with a pH optimum of 5.5. This enzyme may participate in the degradation of ribosomal proteins and histones synthesised in excess of those bound by rRNA and DNA [9]. Despite the fact that proteolytic activity is absent in avian erythrocytes at pH 7 [10] work on nuclear proteins from this source has been carried out in the presence of the serine protease inhibitor PMSF [3]. Since purification of histone pairs via protamine displacement [2], frequently used for nucleosome reassembly studies, is carried out at pH 5, where proteolysis occurs in avian erythrocytes [10], we have investigated the nature of this proteolytic activity to find suitable inhibitors. Properties of the protease which lend themselves to steps in a purification procedure for this enzyme are also presented.

2. Methods

Nuclei were isolated from washed chicken erythrocytes by digitonin lysis and crude chromatin prepared subsequently by washing with 10 mM citrate 150 mM NaCl (pH 7.4) until the supernatant was free of proteins ($A_{280} < 0.1$). The chromatin pellet was resuspended in 10 vol. 50 mM Tris, 15 mM $MgCl_2$ (pH 7.4) containing 2.3 M sucrose and centrifuged for 70 min at 44 000 × *g*. Proteolytic activity was extracted from the sucrose pellet with 10 mM triethanolamine, 0.25 M NaCl (pH 7.4).

Qualitatively proteolytic activity was assayed at 37°C by incubating equal volumes of the sample dialysed against 0.4 mM triethanolamine, 150 mM NaCl (pH 7.4) and total acid-extracted chicken erythrocyte histones at 2 mg/ml in 100 mM acetate, 150 mM NaCl (pH 5.0). After 16 h the reaction was stopped by the addition of SDS–PAGE sample application buffer and incubating for 1 min at 100°C before being applied to an SDS–polyacrylamide gel with a 5% stacking and a 20% separating gel [11]. Total histones were prepared as substrates by acid extraction of chicken erythrocyte nuclei.

Proteolysis was assayed more quantitatively by using radioactively labelled histone H2A as a substrate. Histone H2A was purified from acid-extracted histones by gel filtration on Biogel P60 [12] and then reductively methylated as in [13]. After labelling, H2A was separated from the products of the reaction by Sephadex G-25 chromatography in water before being freeze-dried. The lyophilate was dissolved in 100 mM acetate, 150 mM NaCl (pH 5.0) at 1 mg/ml. (Further details of the assay are given in the legend to fig.1b.)

Chemical inactivation of the proteolytic activity with diazo-acetyl-D,L-norleucine methyl ester (DAN) and Cu^{2+} or with 1,2-epoxy 3-(*p*-nitrophenoxy) propane (EPNP) were carried out as described for cathepsin D [14]. Pepstatin, chymostatin and leupeptin

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[15] were obtained from The Peptide Institute, Osaka. β-Glucoronidase and hexosaminidase were assayed by fluorometrically measuring the cleavage at 37°C of 4-methylumbelliferyl-β-D-glucoside and 4-methylumbelliferyl-*N*-acetyl-β-D-glycosaminide respectively in 100 mM acetate buffer (pH 4.5).

3. Results and discussion

Light microscopy of a stained smear of washed chicken erythrocyte nuclei failed to demonstrate the presence of any nuclei of other origin in a sample of >2500 nuclei. Extraction of the proteolytic activity from crude chromatin was found to be complete with 10 mM triethanolamine, 0.25 M NaCl (pH 7.4). Minimal proteolytic activity was found on re-extraction of nuclei pre-extracted with the above buffer with buffers of increased salt concentration up to 2 M.

Assaying histone degradation electrophoretically demonstrated that of the histones, H2A is more rapidly degraded (fig.1a) than the other core histones with histones H1 and H5 remaining largely intact. The proteolytic activity releases radioactive peptides from tritium-labelled histone H2A (fig.1b).

The protease(s) present in the nuclear extract precipitate between 0.3–0.6 saturation with ammonium sulfate and subsequently elute from a molecular sieve column with an elution volume corresponding to $M_r \sim 39\,000$ (fig.2a). This activity binds to an anion exchange column and can be separated from inactive proteins by elution with a salt gradient (fig.2b).

Through these steps, a purification of the proteolytic activity of 200 times is achieved (table 1).

At 150 mM NaCl, the protease is active without an optimum between pH 2–5 but is effectively inhibited at pH > 5.5 (fig.3a). At pH 5 variation of [NaCl] markedly affects the activity of the enzyme with a maximum at 0.8 M NaCl (fig.3b). To characterise the enzyme more closely the effect of various proteolytic inhibitors was investigated. Only pepstatin [15] is an effective proteolytic inhibitor at low concentration whereas chymostatin [15] has an inhibitory effect only at 100 μg/ml (fig.4). This property together with

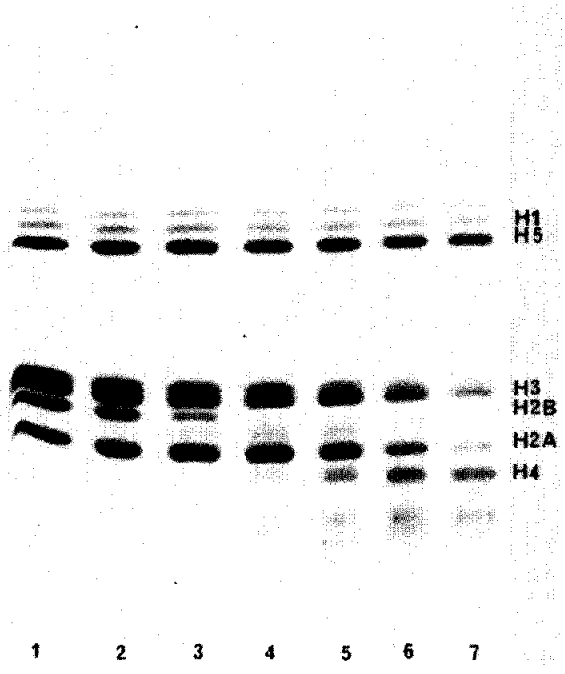

Fig.1a. Time dependence of histone degradation assayed electrophoretically by SDS–PAGE. The reaction was stopped at the following times: 0 min (lane 1, left); 2, 5, 10, 20, 40 and 60 min (lanes 2–7).

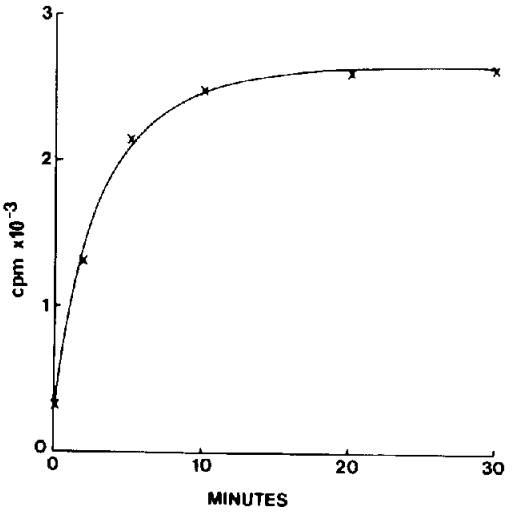

Fig.1b. Time dependence of release of 5% perchloric acid (PCA)-soluble peptides from [^{3}H]H2A. Equal volumes of [^{3}H]H2A in 100 mM acetate, 150 mM NaCl (pH 5) and the sample in 0.4 mM triethanolamine, 150 mM NaCl were incubated at 37°C. Aliquots of 20 μl were withdrawn at the stated time intervals and the reaction stopped by the addition of 100 μl core histones in 50 mM acetate, 150 mM NaCl (pH 5) as a carrier and PCA to 5%. After centrifugation at 12 000 × *g* for 5 min the radioactivity in an aliquot of the supernatant was determined by liquid scintillation counting.

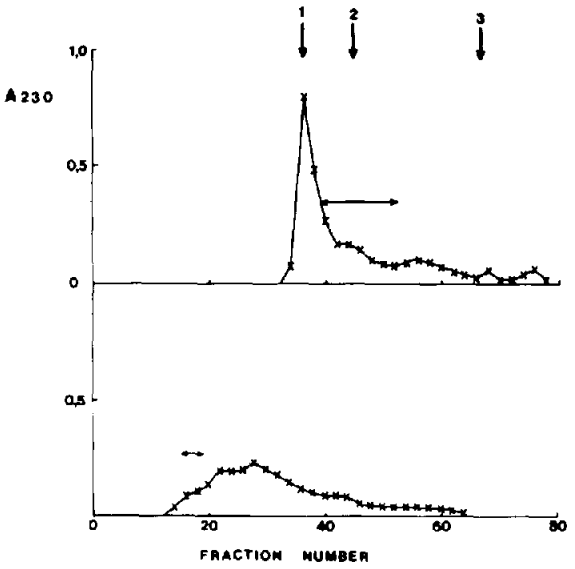

Fig.2. Enzyme purification by molecular sieve chromatography on Biogel P60 (top) and by ion-exchange chromatography on DEAE 52 (bottom). The 10 mM triethanolamine, 0.25 M NaCl (pH 7.4) extract of chromatin was subjected to ammonium sulphate fractionation between 0.3 and 0.6 saturation. The material precipitating at 0.6 saturation ammonium sulphate was dissolved in 10 mM triethanolamine, 150 mM NaCl and applied to a P60 column (1 m × 2.5 cm) in the same buffer. The samples containing proteolytic activity (⟷) were pooled, dialysed against 0.4 mM triethanolamine (pH 7.4) and applied to a DEAE 52 column (1 × 10 cm) equilibrated with the same buffer. After washing with 2 column vol. buffer, the proteolytic activity was eluted with a 0–0.5 M salt gradient in the same buffer. The proteolytic activity was found to be eluted by ~0.15 M salt. The tubes containing activity were pooled and used for characterising the enzyme: (1–3) peak tube of elution of the M_r-markers albumin, ovalbumin and myoglobin.

Table 1
Purification of the proteolytic enzyme

Step	Activity	Purification	Recovery (%)
Triethanolamine (10 mM), NaCl extract (0.25 M)	0.6	1	100
Ammonium sulphate fractionation	2.52	4.2	62
Gel filtration on Biogel P60	17.7	29	49
DEAE-52 ion-exchange chromatography	121.2	199	21

Activity measured in radioactivity released into the supernatant from tritium-labelled H2A . min^{-1} . mg $protein^{-1}$. Purification and recovery refer to overall purification and recoveries

the abolition of activity at pH < 5 suggests that the enzyme extracted from erythrocyte nuclei is an aspartate protease. Accordingly, the effects of the aspartate protease inhibitors diazo-acetyl-D,L-norleucine methyl ester and 1,2-epoxy 3-(*p*-nitrophenoxy) propane were investigated (fig.5). Aspartate proteases

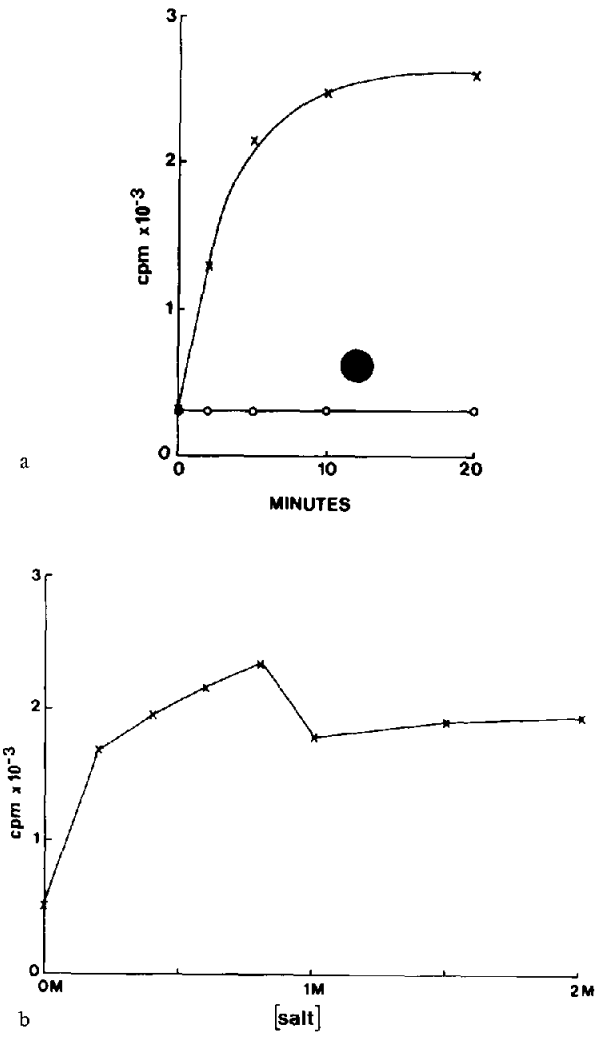

Fig.3. Effect of pH and NaCl concentration on proteolytic activity. (a) The same sample was assayed (cf. fig.1b) in 50 mM acetate, 150 mM NaCl buffer at 0.5 pH unit intervals between pH 2–6. Identical activity was found between pH 2–5 (×——×). The reaction was found not to proceed at pH ⩾ 5.5 (○——○). (b) The same sample was assayed in 50 mM acetate buffer (pH 5) varying the salt concentration. The assay was stopped after 10 min and the radioactivity released into the supernatant determined.

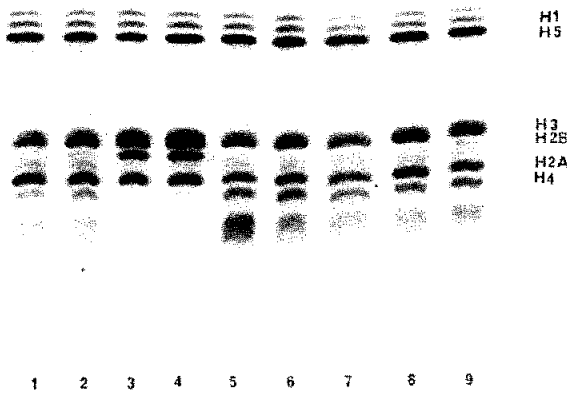

Fig.4. Electrophoretic assay of histone degradation in the presence of inhibitors. The assay was carried out for 16 h at 37°C. The following inhibitors were added (final concentrations in the assay mixture shown): (1) (left) leupeptin 100 μg/ml; (2) chymostatin 10 μg/ml; (3) chymostatin 100 μg/ml; (4) pepstatin 10 μg/ml; (5) bisulphite 0.2 M; (6) PMSF 10 mM; (7) EDTA 5 mM; (8) Ca^{2+} 5 mM; (9) no addition.

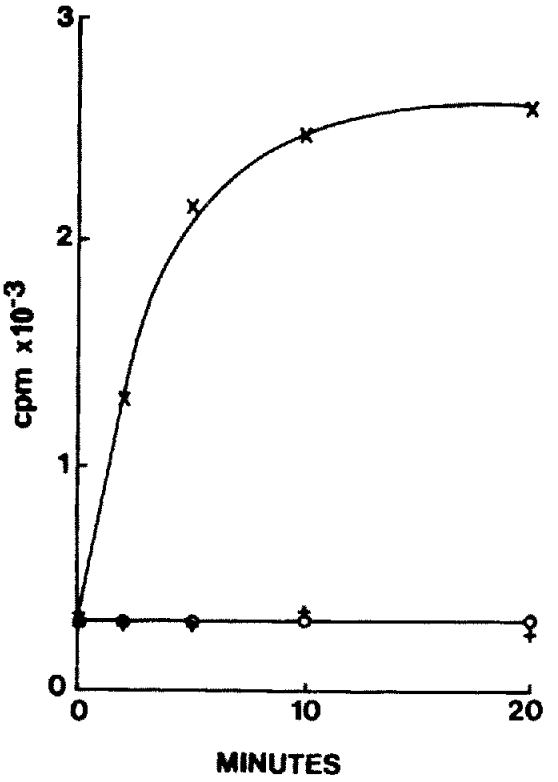

Fig.5. Effect of chemical inactivation with DAN and EPNP. Proteolytic activity was assayed before (x——x) and after incubation with EPNP for 6 h at 21°C (o——o) or in the presence of 5 mM Cu^{2+} (+——+). Dialysis against Cu^{2+}-free buffers failed to restore the activity.

have 2 aspartate residues in the active site of which 1 must be protonated and 1 deprotonated for activity [16]. The effect of pH on the enzyme (fig.3a) indicated that the pK of 2 sidechains was <2 and 5–5.5, respectively. EPNP, which inactivates aspartate proteases by binding to both aspartate residues [17] was found to inhibit the now described enzyme. We were unable to unequivocally demonstrate inactivation of the enzyme by DAN, since Cu^{2+}, required to form the DAN–Cu complex inactivating the protonated aspartate residue [18], were found to irreversibly inactivate the enzyme. Cu^{2+} have been shown [19] to bind to hog pepsin, thereby inducing a conformational change. Possibly this step is irreversible since we found that dialysis against Cu^{2+}-free buffers did not restore the activity of the histone-degrading enzyme.

The enzyme we have isolated appears to be most active in the presence of 0.8 M salt although the change in activity might reflect the changing conformation of the histone with the salt concentration.

Aspartate proteases of other than bacterial origin are known to occur in the stomach (pepsin), the kidney (renin) and in lysosomes (cathepsin D) (for review [16]). We therefore investigated as to whether lysosomal contamination was the source of the proteolytic activity. Controversy exists as to whether chicken erythrocytes contain lysosomes. In an ultrastructural study of avian erythrocytes [20] lysosomes were not reported present but the presence of lysosomal bodies containing acid phosphatase have been reported in [21]. We have found appreciable quantities in chromatin of the 2 lysosomal enzymes β-glucoronidase and hexosaminidase namely 62 μmol . min^{-1} . mg^{-1} and 1.4 nmol . min^{-1} . mg^{-1} protein, respectively. The now identified aspartate protease present in chromatin is thus in all probability the result of lysosomal contamination. A similar enzyme appears to be present in physarum nuclei [22]. To avoid proteolytic histone degradation in chicken erythrocyte histone preparations either the inhibitor pepstatin should be used or applications should be executed at pH > 5.5.

Acknowledgements

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