LYSOZYME (MURAMIDASE) IN ISOLATED CHICK OVIDUCT NUCLEI

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Summary: We have recently observed lysozyme (muramidase) in histone fractions prepared from isolated chick oviduct chromatin. This communication contains a quantitation of the specific activity of nuclear lysozyme. The time course of appearance of nuclear and cytoplasmic lysozyme during hormonally induced tissue differentiation is shown. These and other results suggest that nuclear lysozyme is cytoplasmic in origin and has translocated to the nucleus of the tissue. Conditions are described for the satisfactory extraction of lysozyme from chromatin prepared from nuclei.

INTRODUCTION

Diethystilbestrol (DES) injected into immature chicks results in differentiation of the primative oviduct mucosa into tubular gland, goblet, and ciliated columnar cells. Continued injections of the hormone result in the production of the major egg-white proteins (ovalbumin, conalbumin, ovomucoid, and lysozyme) in the tubular gland cells (1). These are then secreted into the egg during its passage through the oviduct. The localization of lysozyme in cell fractions has not been studied in detail since egg white is an abundant source of this enzyme. Our observation of nuclear lysozyme in oviduct tissue, which is in apparent conflict with the general belief that lysozyme is a cytoplasmic enzyme, prompted us to examine the finding in greater detail.

METHODS

Highly purified nuclei were isolated from the oviducts of female Leghorn chicks using the hexylene glycol buffer previously reported (2). Oviducts were cleaned of vascular and connective tissue, minced, and homogenized in 15 ml/g oviduct of buffer HPC-1 (0.5 M hexylene glycol (Eastman), 1 mM piperazine-N, N' bis (2-ethane sulfonic acid) sodium salt monohydrate (Calbiochem) pH 7.0, and 1 μM $CaCl_2$). The homogenate was filtered through 4 layers of cheesecloth and

then a layer of organza, and centrifuged for 5 minutes at 900 x g. The pellets were washed 3 times in buffer HPC-1 and suspended in 10 ml/g oviduct of 2.0 M sucrose, 0.1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5 and centrifuged at 25,000 x g for 40 min. The resulting pellet is the HPC nuclear fraction. For comparison, in a single instance, nuclei were prepared by a modification of the sucrose-TKM method (3). In this case oviducts were homogenized (20 ml/g tissue) in 0.5 M sucrose-TKM (2 mM $MgCl_2$, 25 mM KCl, 5 mM Tris-HCl, pH 7.4) The homogenate was filtered through four layers of cheesecloth followed by a single layer of organza and centrifuged at 3,000 x g for 5 min., resuspended in 2.2 M sucrose-TKM and centrifuged at 15,000 x g for 45 min. The resulting pellet is the TKM nuclear fraction.

Chromatin was isolated by homogenizing the HPC nuclear fraction in 10 volumes (ml/g oviduct) of 0.14 M NaCl which were then centrifuged at 3,000 x g for 10 min. This was repeated and the pellet was homogenized in 10 volumes of 0.3 M NaCl and centrifuged as above. The pellet was then washed twice in 10 volumes of 0.01 M Tris-HCl, pH 7.5, and centrifuged at 7,000 x g for 10 min.

Histone was prepared from nuclei by extraction with 0.4N H_2SO_4 and overnight precipitation in the presence of absolute ethanol.

Acetic acid-urea (2.5M) polyacrylamide (15%) gel electrophoresis was carried out as previously described (4) using calf histone (Sigma) and 3X crystalled egg lysozyme (Sigma) as standards. Gels were stained with Amido Schwartz and destained by diffusion.

Enzymatic activity of lysozyme was measured using a Lysozyme Assay Kit (Worthington) and compared to 3X crystallized lysozyme standard (Sigma).

Protein was estimated by the method of Lowry (5) using calf thymus histone (Sigma) standard.

Radioactively iodinated proteins were prepared by a solid state lactoperoxidase-glucose oxidase coupled system (6,7) using ^{125}I obtained from New England Nuclear.

RESULTS

Because our standard procedure for the preparation of nuclei relies on a buffer of low ionic strength (1 μM $CaCl_2$) and low dielectric constant (provided by 0.5 M hexylene glycol) we considered the possibility that the presence of lysozyme in the nuclear fraction resulted from these conditions or from a loose association which could be disrupted by detergents.

Figure 1 shows polyacrylamide gels of the histone fraction prepared from nuclei isolated by different methods. All histone fractions examined contain a band migrating faster than histone f2a1 (IV) at the same R_f as authentic lysozyme.

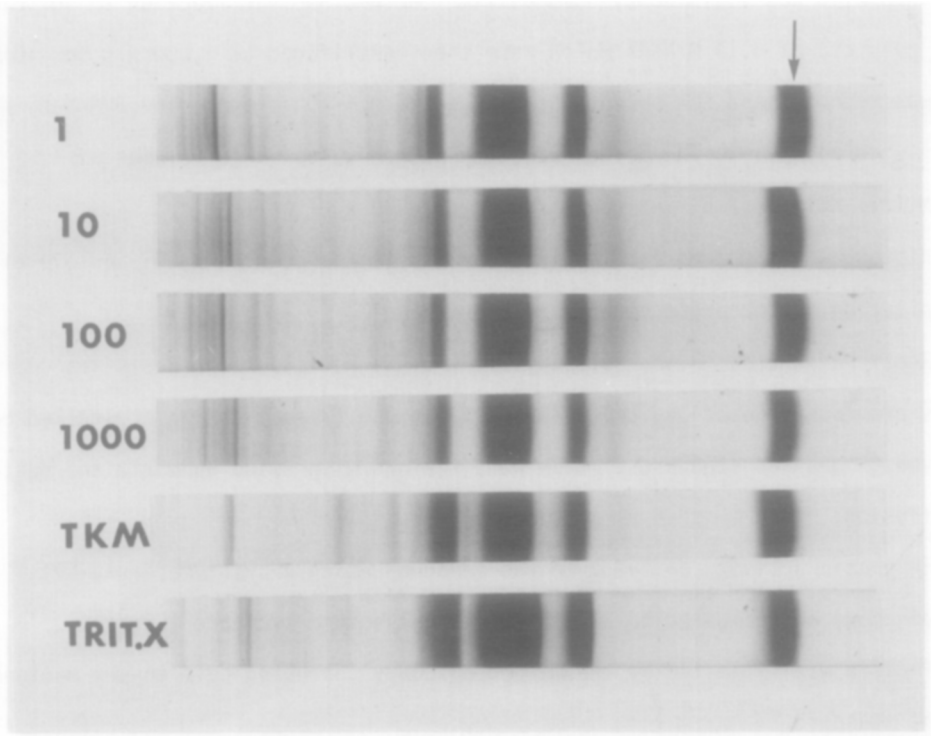

Figure 1. Effect of Isolation Buffers and Detergent Washing on the Presence of Lysozyme in the Histone Extracted from Chick Oviduct Nuclei. Nuclei were prepared by our standard procedure (2) or by a modification in which the concentration of $CaCl_2$ was altered to contain either 10 μM, 100 μM, or 1,000 μM. TKM nuclei were prepared as described in Methods. Detergent washed nuclei were prepared by washing nuclei in 1% Triton X-100, a procedure believed to remove the outer membrane of the nuclear envelope (3).

Moreover the specific activity of lysozyme from all methods of isolation is fundamentally the same (see Table I). These results suggest that the presence of lysozyme in the nucleus is: (1) not an artifact of low ionic strength isolation; (2) not resultant from the use of hexylene glycol in isolation buffers; and (3) not removed by washing in Triton X-100.

Because of the distinctive R_f of this protein, we were able to isolate it by preparative gel electrophoresis of the histone fraction. In addition to showing the presence of lysozyme activity, the amino acid composition was determined (see Table II) and found to be similar to values reported for chick type lysozyme (8).

We have additionally found that lysozyme is present (8.9 U/μg protein) in the histone fraction prepared from chromatin which has not been washed with 0.3 N NaCl. Lysozyme can no longer be detected (<0.01 U/μg) if the salt wash is included.

In order to determine if uptake of lysozyme into the nucleus occurred during preparation of nuclei, a small amount of (<0.03 mg) of [^{125}I] lysozyme

TABLE I

Specific Activity of Lysozyme in Histone Fractions Prepared from Isolated Nuclei

METHOD *	UNITS OF LYSOZYME ACTIVITY PER μg PROTEIN ▲
Standard Procedure	9.19
10 μM $CaCl_2$	10.38
100 μM $CaCl_2$	8.89
1000 μM $CaCl_2$	14.48
TKM	10.29
Trition X-100 Washed	9.21

* Nuclei were prepared as described for Figure 1. ▲ Lysozyme specific activity was determined in the histone fraction as described in Methods.

TABLE II

Amino Acid Composition of Nuclear Lysozyme*

Amino Acid	Percent▲	Amino Acid	Percent▲
Lysine	6.3	Alanine	10.2
Arginine	9.8	Valine	4.3
Aspartic Acid + Asparagine	19.0	Methionine	0.7
Threonine	4.9	Isoleucine	6.5
Serine	6.9	Leucine	7.2
Glutamic Acid + Glutamine	4.7	Tyrosine	2.0
Proline	3.5	Phenylalanine	1.9
Glycine	12.0		99.9

*Nuclear lysozyme was isolated by preparative gel electrophoresis of the histone fraction. Acid urea gel is described in Methods.
▲Values are % moles present in each amino acid.

(>6 x 10^6 cpm/mg) was added to the tissue homogenization mixture (3g oviduct) from which a nuclear pellet was isolated. Approximately 7.5% of the total counts were later recovered associated with the nuclear fraction. The remainder appears in washes. When a similar experiment was done with [^{125}I]ovalbumin no counts above background were found in the nuclear fraction. Further evidence for the cytoplasmic origin of nuclear lysozyme is the similar time course of the appearance of nuclear and cytoplasmic lysozyme during hormonally induced differentiation (Fig. 2). Nuclear and cytoplasmic lysozyme activity both increase after the third day of estrogen stimulation and continue to rise until a plateau value is reached (day nine). After the initial stimulation, nuclear activity (U/µg protein) is consistently higher than cytoplasmic values (U/mg protein). When hormone injections are discontinued for 14 days, both nuclear and cytoplasmic activities return to unstimulated levels.

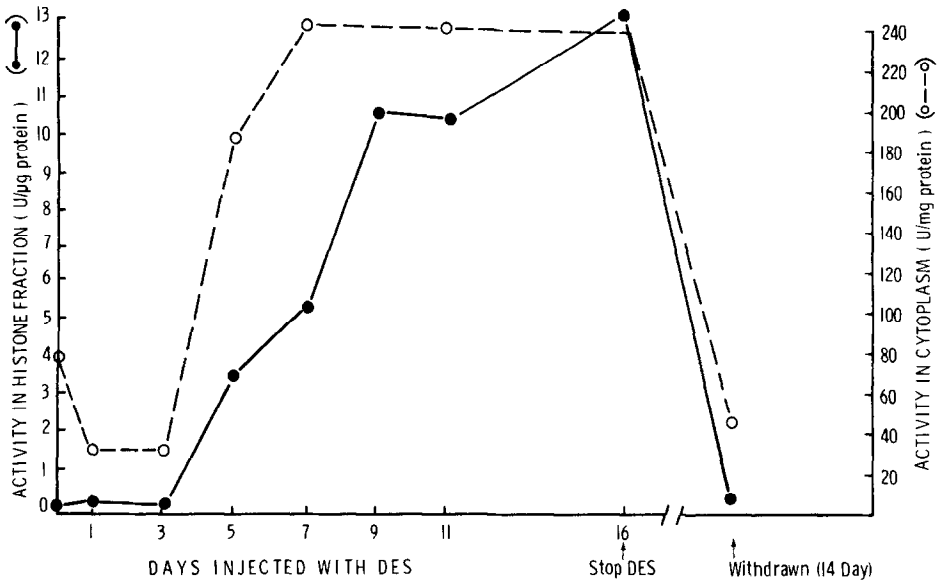

Figure 2. Specific Activities of Nuclear and Cytoplasmic Lysozyme. Histones were extracted from nuclei isolated from oviducts of chicks stimulated with DES for 0, 1, 3, 5, 7, 9, 11, or 16 days or from animals stimulated for 14 days and then withdrawn from hormone for 14 days. These fractions were assayed for specific activity of lysozyme. The post nuclear supernatant (5 min. 1,000 x g) was similarly assayed.

DISCUSSION

We report above the presence of lysozyme in the highly purified nuclear fraction of estrogen-stimulated chick oviduct. Evidence is presented that a portion of the nuclear lysozyme is cytoplasmic in origin. Paine and Feldherr (9) demonstrated that fluorescently labeled lysozyme micro- injected into the cytoplasm of oocytes concentrated in the nucleus within 3 minutes. While the mechanism and significance of this translocation is not known, the binding of lysozyme to DNA *in vitro* has been studied as a model system for the interaction of DNA with basic proteins (10).

Our data are consistant with a cytoplasmic-nuclear translocation and suggest that the use of high salt in the preparation of chromatin is essential to extract proteins associated, though not tenaciously, with the DNA.

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