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Original Research Article

Isolation and Characterization of Total Histones from Chicken Erythrocytes: A Simpler Method Using Detergent

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ABSTRACT

Histones are highly conserved small basic proteins that are positively charged at neutral pH. They associate with the DNA to form a polymer called chromatin. Many studies have focused on the isolation of histones from tissues and cell lines. The present study reports the isolation of good-quality histones from chicken erythrocytes by using a high-speed centrifugation method through a sucrose gradient. The method described in the present study is simpler and is a deviation from the usual requirement of an ultracentrifugation step for the purification of the histones. The isolated histones were quantified using spectrometric methods and the quality and integrity of the histones were evaluated by a high-resolution SDS 18% PAGE. The isolated histones were found to be homogeneously pure. They contained all the histone subtypes in stoichiometric amounts along with the histone variants. The isolated total histones were subsequently used for routine histone protease assays and they were demonstrated to be a great substrate for these assays. The present study thus describes the chicken erythrocyte total histones isolated from chicken blood (a waste product of the slaughterhouse), by a high-speed centrifugation method, evaluation of their quality and their usefulness as a substrate for histone-specific protease assays.

Keywords: Chicken erythrocyte histones, Histone isolation, Chromatin & Histone specific proteases

1. INTRODUCTION

The eukaryotic DNA which is ~2 meters long is huge enough to fit inside the tiny nucleus. To enable this, the DNA forms a macromolecular complex by being associated with histone and non-histone proteins to form a dynamic polymer named chromatin (Purohit & Chaturvedi, 2016; Vaquero et al., 2003). The chromatin contains repeating units called nucleosomes. Each nucleosome is made up of 146/147 bp of DNA, wrapped 1.75 turns around a core histone octamer. The core histone octamer is composed of 2 copies each of H2A, H2B, H3 and H4. The entry and exit points of the octamer in the nucleosomes are sealed by linker histone H1 (Richmond & Davey, 2003). Histones are highly conserved proteins having a net positive charge at neutral pH. They have core domains mainly made up of α-helix and unstructured N/C termini. Apart from these canonical histones, many histone variants are also reported (Maze et al., 2014). Chicken erythrocytes have a major histone variant, histone H5.

The chromatin is subjected to remodelling by ATP-dependent chromatin remodelers or by many reversible posttranslational modifications of the histones (Clapier & Cairns, 2009; Panigrahi et al., 2003; Singh et al., 2023; Tyagi et al., 2016; Vaquero et al., 2003. Site-specific cleavage of the N- or C-termini of histones has also been reported as another type of irreversible post-translational modification (Dhaenens et al., 2015; Purohit et. al., 2012). By these chromatin remodelling mechanisms, histones act as regulators of all nuclear events such

as replication, transcription, repair and recombination. Recently, it has been shown that histones, in addition to their conventional localisation in the nucleus, are also found in the cytoplasm, extracellular vesicles, and external secretions (Singh et al., 2022). These non-nuclear histones have been proposed to have a role in cytotoxicity and innate immunity.

For assays which use histones as substrates, it is essential to isolate good-quality histones. Histones being rich in lysine and arginine residues are also susceptible to proteolytic degradation by non-specific protease, mostly of cytoplasmic origin, during isolation. Hence for the isolation of histones, it is crucial to purify good-quality nuclei, almost devoid of cytoplasmic contaminations. Various studies have reported histone isolation from different model organisms like Tetrahymena, mice, chicken liver, human blood etc. Histone isolated from these already established methodologies mostly used a sucrose gradient and an ultracentrifugation step with the sporadic use of HPLC (Holt et al., 2021). It usually resulted in low yield and longer time duration for histone isolation.

The present study reports the isolation and characterisation of total histones from chicken erythrocytes. Chicken erythrocytes are nucleated cells. Chicken blood is usually considered a waste of the slaughterhouse. Here, high-speed centrifugation is used in the presence of mild detergent to isolate histone from erythrocytes of chicken blood. The quality of these histones is subsequently evaluated on an SDS 18% PAGE.

2. MATERIALS AND METHODOLOGIES

All the experiments were performed following the guidelines of the Institutional Ethical Committee. Blood was collected from freshly sacrificed chicken in the slaughterhouse, in a tube containing an equal volume of blood collection buffer (2X PBS, 4 mM EDTA, 2 mM PMSF, 100 mM sodium bisulfite). The blood was brought to the lab on ice and immediately processed for the isolation of histones.

2.1. Isolation of nuclei from chicken blood

The nuclei from chicken erythrocytes were isolated by the already established methods for chicken liver (Panda et al., 2011; Panigrahi et al., 2003; Purohit et al., 2013), with minor modifications. Briefly, 50 ml of the chicken blood was filtered through two layers of sterile muslin cloth to remove any tissue debris and centrifuged at 5,000 x g for 20 min at 4°C. The supernatant was discarded and to the pellet, an equal volume of chilled Solution-I [0.34 M sucrose, 15 mM Tris-HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 3 mM $MgCl_2$ (15 mM β-Mercaptoethanol and 1mM PMSF was added just before use)] and was gently mixed with the help of a pipette. To the tube, 10% Triton X-100 was added till a final concentration of 0.1%. It was centrifuged immediately at 5,000 x g for 20 min at 4° C and the supernatant was discarded. The pellet containing lysed blood cells was repeatedly washed 2-3 times as above, with Solution I containing a decreasing concentration of Triton X-100, till the nuclear pellet appeared yellowish/pinkish, indicating the removal of maximum haemoglobin contamination. Subsequently, the nuclear pellet was washed with solution III [0.34 M sucrose, 15 mM Tris-HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 3 mM $MgCl_2$ (15 mM β-Mercaptoethanol and 1mM PMSF was added just before use)] three times by centrifugation, as above.

2.2. Estimation of the chicken erythrocyte nuclei

The nuclei isolated from the chicken erythrocytes were estimated in terms of their DNA content by an already-established method (Panda et al., 2011). Briefly, an aliquot of the washed nuclei in Solution III was disrupted in 1 ml of 5 M Urea 2 M NaCl solution and the absorbance at 260 nm was recorded using a Shimadzu 1600 UV-VIS 1900 spectrophotometer. Here, 5 M Urea 2 M

NaCl solution acted as a blank. An A_{260} of 1, equivalent to 50 µg/ml of DNA was taken as standard for calculating the DNA content of the chicken erythrocyte nuclei.

2.3. Isolation of total Histones from chicken erythrocyte nuclei

Histones were isolated from the purified chicken erythrocyte nuclei by standard methods (Hoffman & Chalkley, 1978), with minor modifications. Briefly, the washed nuclear pellet after the 3rd wash in Solution III was resuspended in wash buffer [10 mM Tris-HCl pH 7.4, 75 mM NaCl, 24 mM EDTA, (15 mM β-Mercaptoethanol and 0.2 mM PMSF was added freshly)], at a concentration of 1 mg/ml DNA and was centrifuged at 10,000 x g for 15 min at 4°C. The pellet was resuspended in resuspension buffer [10 mM Tris-HCl pH 7.4 with freshly added 15 mM β-Mercaptoethanol and 0.2 mM PMSF] at a concentration of 1 mg/ml DNA, as above and placed on ice. To the nuclei suspension, 2 N H_2SO_4 was added dropwise with intermittent swirling, to the final concentration of 0.4 N H_2SO_4. The histones were allowed for acid extraction on ice, for 1 h with intermittent swirling. The acid-extracted histones were centrifuged at 10,000 x g for 30 min at 4°C to pellet the genomic DNA and the supernatant containing the histones was collected. Four volumes of chilled acetone were added to the supernatant and the histones were precipitated overnight at -20°C. The next day, the precipitated histones were collected by centrifugation at 10,000 x g at 4°C. The pellet, containing histones was washed twice with chilled acetone, as above. The purified histone pellet was air-dried on ice and processed further.

2.4. Estimation of the isolated Histones

The air-dried histone pellet was dissolved in sterile water on ice. The dissolved histones were estimated spectrophotometrically by taking absorbance at 230 nm (Sollner-Webb et al., 1976). An A_{230} of 3.3, equivalent to 1 mg/ml of histone was taken as standard. The final concentration of the histones was adjusted to 6 µg/ml and stored at -20°C for subsequent assays.

2.5. Separation of the Chicken Erythrocyte Histones on a High-resolution SDS 18% PAGE

The histones were separated on a high-resolution SDS 18% PAGE and subsequently stained with coomassie brilliant blue (CBB) R250 staining by already established methods (Laemmli, 1970) with minor modifications (Purohit et al., 2017). Briefly, 6 µg histone samples were mixed with one-fifth volume of 5x Laemmli buffer [1x composition (0.0627 M Tris -HCl (pH 6.8), 1% SDS, 10% glycerol, 0.0025% bromophenol blue, and 1% β-Mercaptoethanol)] and boiled for 3 min in a boiling water bath and chilled immediately on an ice bath. These samples were loaded on to a high-resolution SDS 18% PAGE with resolving gel [18% polyacrylamide (30:0.8 acrylamide: bis-acrylamide), 0.75 M Tris HCl (pH 8.8), 0.1% SDS, 0.07% APS and 0.06% TEMED], stacking gel [5% polyacrylamide (30:0.8 acrylamide: bis-acrylamide), 0.125 M Tris HCl (pH 6.8), 0.1% SDS, 0.07% APS and 0.06% TEMED] and with electrode buffer (25 mM Tris base, 192 mM glycine, and 0.1% SDS). Electrophoresis was carried out at a constant current of 20 mA, till the loading dye reached the end of the resolving gel. The gel was stained with staining solution (45% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250) overnight on a rocker and destained using the destaining solution (10% acetic acid and 45% methanol).

3. RESULTS

3.1. High-speed centrifugation is a more efficient method of isolation of nuclei from chicken erythrocytes

In the present study, the isolation of the nuclei from chicken erythrocytes has been optimised by using a low amount of Triton-X 100, a detergent that lysed the cell membrane of the

erythrocytes, while the nuclear membrane remained intact. The major contaminant of the erythrocytes (in the context of histone isolation) was haemoglobin, which was efficiently removed by repeated washing of the chicken erythrocyte nuclei with Triton-X 100 (Figure 1a). Further, in the present study, the chicken erythrocyte nuclei were isolated by using 0.34 M sucrose gradient, using a high-speed centrifuge at 5000 x g. This method contrasts with the majority of the histone isolation methods from tissues, where usually a 2.1 M sucrose gradient is used and the nuclei are pelleted by an ultracentrifugation step (Dean et al., 1973). The chicken erythrocyte nuclei isolated using high-speed centrifugation, in the presence of the detergent (Triton X-100) were found to be intact and fairly pure. The purity and morphological integrity of the isolated nuclei were routinely monitored by visualizing them under a phase contrast microscope. The isolated nuclei were intact, round-shaped, with clear nucleoli, and were also almost free from cytoplasmic contaminations (Figure 1b).

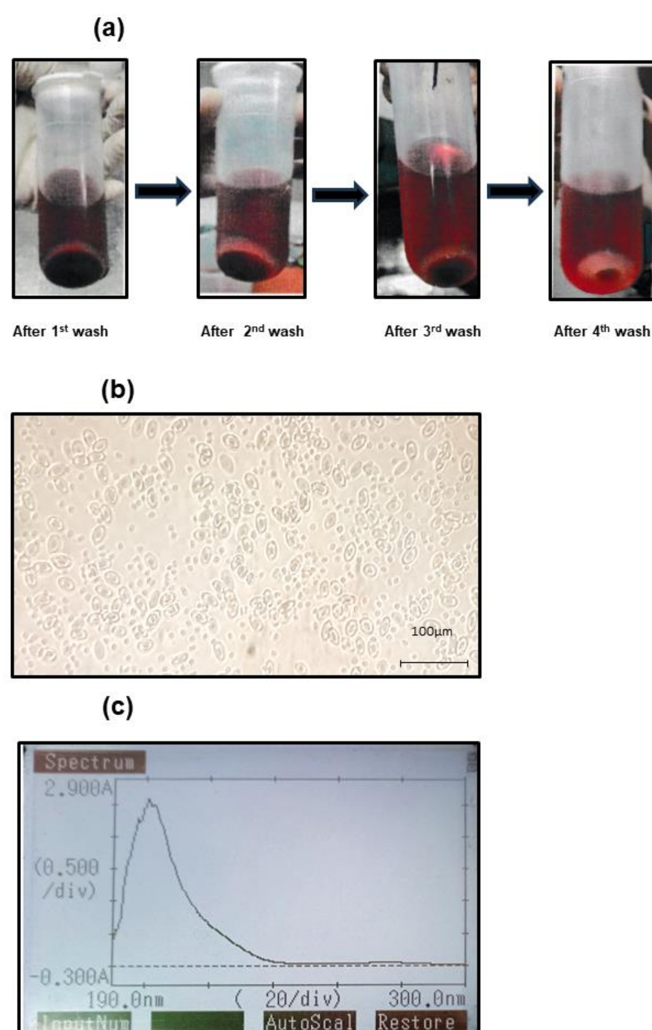

Figure 1. Isolation of chicken erythrocyte total histones (CETH). **(a)** The chicken blood collected in an equal volume of blood collection buffer was centrifuged at 5000 x g. The pellet was resuspended with an equal volume of Solution-I containing 0.1% Triton X-100 and centrifuged as above. The pellet was subsequently washed with reducing volumes of Triton X-100 by centrifugation as above. Finally, the obtained nuclei were resuspended in Solution III and washed as above. **(b)** The purified nuclei pellet from chicken blood obtained after the washes with solution III was resuspended in Solution III, smeared onto a glass slide and observed under a phase contrast microscope to visualise the nuclei. **(c)** The chicken erythrocyte total histones (CETH) were dissolved in a minimum amount of sterile distilled water containing 0.1M PMSF and the absorption spectra was measured in the range of 190 nm to 300 nm to evaluate the purity.

Simultaneously, the chicken erythrocyte nuclei were estimated in terms of their DNA content by measuring the absorbance of an aliquot at 260 nm. The DNA content of the chicken erythrocyte nuclei was calculated by considering an OD of 1 equivalent to 50 µg/ml of DNA. The amount of nuclei isolated from 200 ml of chicken blood is represented in Table 1.

The acid extraction method was utilised for the isolation of histones from erythrocyte nuclei. The extracted histones were acetone precipitated, air-dried, and dissolved in sterile water. The isolated histones were quantified by measuring an absorbance at 230 nm. The amount of histone isolated from the above erythrocyte nuclei is tabulated in Table 1. Further, to verify their purity, these histones were subjected to a spectrophotometric scan in a range of 190 nm to 300 nm (Figure 1c). The absorption maxima were recorded at 230 nm and no second peak was observed in the scan range (no peak even at 280 nm, usual for all proteins except histones), suggesting that the isolated histones had no significant protein contamination and were fairly pure.

Table 1. Spectroscopic estimation of isolated DNA and histones. The isolated nuclei were estimated for their DNA content by taking an absorbance at 260 nm and the isolated histones were estimated at 230 nm.

The volume of Chicken Blood	Estimated DNA (A_{260}) (mg)	Estimated Histones (A_{230}) (mg)
200 ml	154.8 mg	155.75 mg

3.2. Analysis of the chicken erythrocyte total histones by SDS-PAGE

The chicken erythrocyte total histones (6 µg) were denatured and separated on an SDS 18% PAGE followed by CBB staining (Figure 2 left panel). The results revealed that the isolated histones contained all the histone subtypes in stoichiometric amounts.

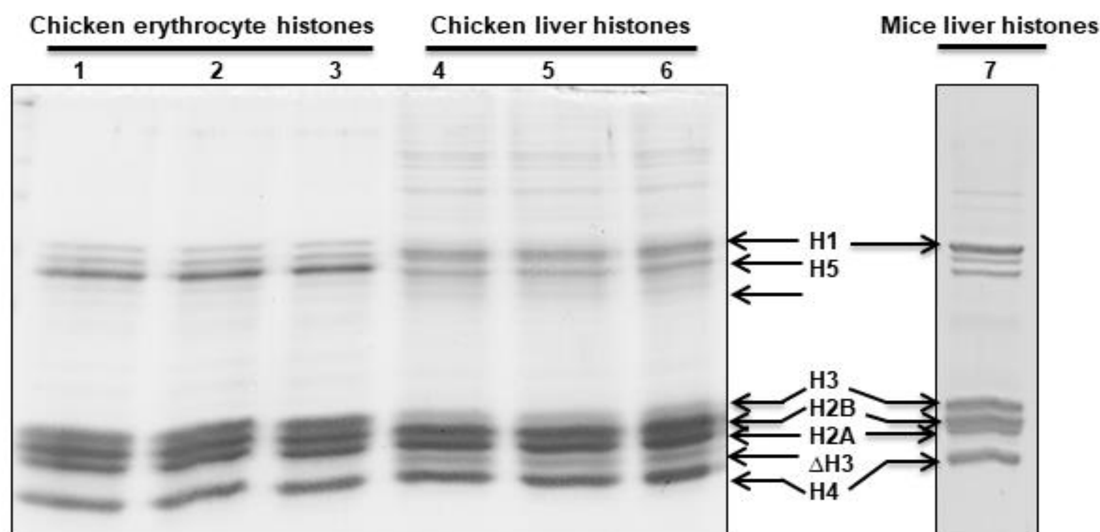

Figure 2. Qualitative analysis of the chicken erythrocyte total histones (CETH), chicken liver total histones (CLH) and mice liver total histones (MLH) by SDS 18% PAGE. 6 µg each of chicken erythrocyte total histones, chicken liver total histones and mice liver total histones were resolved on SDS 18% PAGE, and stained with Coomassie brilliant blue staining and visualised to evaluate the quality of histone subtypes.

Further, the canonical histone subtypes and the H5 variant were intact and were not proteolytically degraded. Degradation is a very common problem during histone isolation, at least while being isolated with the use of a high-speed centrifugation step, due to the retained cytoplasmic protease contaminations in the nuclear fraction. However, in the present study, even if the nuclei were not purified using an ultracentrifugation step, the obtained histones were of good quality, almost without other protein contamination. Adopting a similar method, histones from chicken liver and mice liver, were also isolated (Figure 2 right panel). Though, it can be clearly stated that in terms of quality and contaminating protein bands, the chicken erythrocyte histones were of superior quality compared to chicken liver and mice liver histones. In the case of mice liver, the cleavage of histone H3 and the appearance of a faster migrating delta H3 (ΔH3) was reported (Purohit et al., 2017).

3.3. Histone Protease assay using Chicken Erythrocyte Total Histones (CETH) as substrates

We have previously purified and characterised the histone H3 and the H2A-specific proteases (H3ase and H2Asp) from chicken liver nuclear extract (Panda et al., 2013; Panda et al., 2011; Purohit et al., 2013: Purohit et al., 2024). While establishing the assay system, mice liver total histones or chicken erythrocyte total histones were used as substrates for these assays. According to the observations made, total histones obtained from commercial sources did not qualify as great substrates for these assays due to degradation and other contaminations present in them. Further, in these protease assays, the other histone subtype bands of the total histones (such as H2B and H4) acted as inadvertent internal controls to depict the histone subtype specificity of the proteases. Hence, for these protease assays, even bacterially expressed single histones also did not qualify as good substrates.

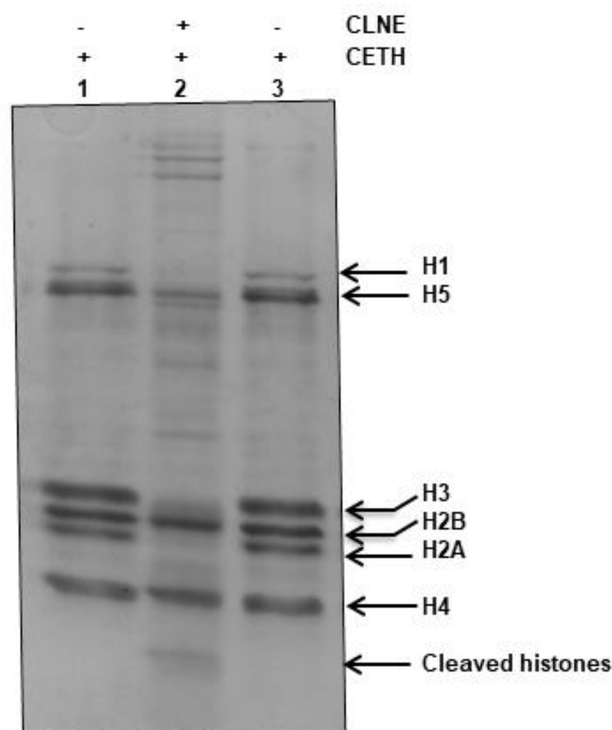

Figure 3: Histone-specific protease assay using the chicken erythrocyte total histones (CETH) as substrates. Histone-specific protease assay was performed by incubating 6 µg of CETH with 1 µg of chicken liver nuclear extract (CLNE) (the source of the H3 and H2A-specific proteases) in the already established assay system. The cleaved histones were resolved on an SDS 18% PAGE and CBB stained. 6 µg of CETH alone, without CLNE acted as the control lanes. Lane 1: CETH; lane 2: CETH+CLNE; lane 3: CETH.

Accordingly, the chicken erythrocyte total histones from the present experiment were used as the substrate for the protease assay by using 1 µg chicken liver nuclear extract (CLNE) in the protease assay system already developed by us. The cleaved histones were subsequently resolved on SDS 18% PAGE and CBB stained. The results revealed that the isolated chicken erythrocyte total histones also acted as good substrates for the histone protease assay (Figure 3). It could be seen that the histone H3 and histone H2A could be efficiently cleaved by the CLNE. Further, partial cleavage of histone H5 also could be observed. It suggested that the chicken erythrocyte total histones isolated through the high-speed centrifugation of the present experiments also posed as a great substrate for histone-specific protease assay.

4. CONCLUSION

The present study explores the need for isolation of total histones from chicken erythrocytes. The optimised method in the present study did not significantly compromise the quality of the histones. Conversely, it significantly enhanced the yields of isolated histones compared to the reported method which used ultracentrifugation steps. Further, the isolated histones could be successfully used for histone protease assay and other chromatin assays.

COMPLIANCE WITH ETHICAL STANDARDS

All the experiments were conducted under the guidelines of the institutional ethical committee.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR’S CONTRIBUTION

Y.R., M.B. and B.S.: performed the experiments and manuscript writing and preparation of table and figures; M.M.C.: experimental designing and suggestions; J.S.P.: experimental designing, manuscript correction and suggestions.

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