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Identification of a novel histone H3 specific protease activity in nuclei of chicken liver

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ABSTRACT

Evolutionary conserved histone proteins play a very important role in the regulation of eukaryotic gene expression by undergoing post translational modifications within the tail regions. However, their role in tissue-specific gene expression and development remains unclear. In this study, we provide evidence for *in vivo* tissue-specific proteolytic cleavage of histone H3 in the liver of adult white Leghorn chickens, which we believe to be regulated by tissue-specific protease activity and epigenetic markers. The cleavage of histone H3 in the liver of adult chickens is very unique, and can serve as a model for studying tissue-specific changes in chromatin organization and gene expression. For the first time, we have identified and partially purified histone H3-specific protease activity that is distinct from histone H3 protease activities recently reported. Together, our data provide evidence of proteolytic processing and identification of protease activity that is specific to histone H3 in the liver of adult chickens, which may be involved in the regulation of gene expression during development, aging, and age-associated diseases.

1. Introduction

The DNA of all eukaryotic genomes is organized into a nucleoprotein complex called chromatin [1]. Chromatin is essential for compacting genomic DNA and plays a primary role in governing DNA accessibility and gene expression. Eukaryotic chromatin is made up of repeating units, called nucleosomes, which contain four types of evolutionary conserved, structural, and regulatory histone proteins. Two molecules each of histone proteins H3, H2A, H2B, and H4 together make an octamer structure, which is wrapped with 146 base pairs of DNA. The composition of chromatin undergoes dynamic changes during processes of DNA replication, transcription, repair, recombination, aging, and apoptosis [2–4]. Importantly, chromatin not only enables the compaction of the genome in limited nuclear space, but also represents a physical barrier to these processes [5,6]. Post-translational modifications (PTMs) of nucleosomal core histones can alter the structure and function of chromatin [7,8], and while the role of PTMs is well established, the significance of histone tail truncation is poorly understood [9–18].

Several studies have shown that activation of gene expression involves transient decondensation of chromatin structures, which is facilitated by specific PTMs within the histone tail domain [8], the exchange of histone variants [19,20], and ATP-dependent chromatin remodeling activities [21]. In addition to these reversible chemical modifications of histone tails, sections of certain histone tails also undergo proteolytic cleavage during the cell cycle, development, viral infection [22], mouse embryonic stem cell differentiation [15], aging [23,24], spermatogenesis, and sporulation [25]. However, the molecular mechanism behind the effect of such modifications on the structure and function of chromatin during aging is not clear. In some cases, however, proteolytic processing, particularly of histone H3 and H1, has been considered to be a physiologically-regulated event [9,10]. For example, in *Tetrahymena*, six amino acids are removed from the NH_2-terminus of histone H3 in transcriptionally silent micronuclei. During viral infection with the foot-and-mouth disease virus, H3 has been reported to be cleaved between Leu20 and Ala21 from the N-terminus [22]. Recently, two important observations have been published regarding histone H3-specific proteolysis. One protein, Cathepsin L, regulates differentiation of stem cells in mammals, and another as yet unidentified factor in yeast regulates the process of sporulation. Cathepsin L has been identified as an enzyme that cleaves the N-terminus of histone H3 during mouse embryonic stem cell differentiation [15]. In yeast (*Saccharomyces Cerevisiae*), a histone H3-specific endopeptidase activity is required for the expression of sporulation phase-specific genes [25]. Thus, cleavage of histone H3 seems to be an evolutionary conserved process, but its biological function remains to be investigated in different organisms. In the present study, we provide evidence for the first time of a tissue-specific histone H3-specific N-terminal cleavage enzyme in

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adult chicken liver, which we believe affects chromatin structure and gene expression during aging, apoptosis, and age-associated diseases. The cleavage of H3 can be catalyzed by the action of a specific protease that is expressed or becomes active during these processes. To identify such an enzyme, we used liver and brain tissue extracts from an adult chicken. Interestingly, we found histone H3- specific protease activity in the nuclear extracts of liver tissue only. In order to prevent contamination of the nuclear extracts with the cytosol, nuclei were purified from adult chicken livers through a 2.1 M cushion of sucrose. Using ammonium sulfate precipitation and various chromatography columns, we succeeded in partially purifying a protease with activity against histone H3, which is different than recently reported H3 proteases. Taken together, our data have identified age-dependent N-terminal proteolytic processing and have identified novel histone H3-specific protease activity in the chicken, which may account for global regulation of gene expression during aging, development, and age-associated diseases. To the best of our knowledge, our findings are novel and have not been previously reported in a chicken model.

2. Materials and methods

White Leghorn chickens were used for the experiments. Tissues (liver, brain, kidney, and erythrocytes) from freshly sacrificed chickens were transported on ice from the slaughter house under approval from the Institutional Biosafety Committee (IBSC). The animals were not sacrificed by the authors of this study. For analysis of histones, tissues were collected from two-day-old (young) and two-month-old (adult) animals. Tissues were immediately washed with cold normal saline and stored at −80 °C. Protease inhibitors, iodoacetamide (I1149), leupeptin (L2884), bestatin (B8385), E-64 (E3132), EDTA (E5134), and recombinant anti-Cathepsin L (L-C2970) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.1. Preparation of histones from chicken liver, brain, kidney and erythrocytes

Histones were extracted from purified nuclei by H_2SO_4 and hydroxyapatite by following a standard procedure as previously described [26]. Nuclei were isolated using a method previously described with some modifications [27]. Briefly, tissues were homogenized using a motor driven Potter–Elvehjem homogenizer in Solution 1 (0.34 M sucrose, 15 mM Tris–Cl pH 7.5, 15 mM NaCl, 60 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA, 15 mM β-mercaptoethanol, and 0.2 mM PMSF) to prepare a 10% homogenate. Triton X-100 was added to the homogenate to a final concentration of 0.25% followed by centrifugation at 5000 rpm for 20 min. Nuclei pellets were washed 3 times with Solution 1 containing 0.34 M NaCl but no detergent. Nuclei were suspended in 10 mM Tris–Cl (pH 7.5), 0.2 mM PMSF, and 15 mM β-mercaptoethanol at a concentration of 0.5 mg DNA/ml. Histones were purified from isolated nuclei by H_2SO_4 extraction and hydroxyapatite chromatography. For hydroxyapatite chromatography, washed nuclei were sonicated in a hypotonic solution (10 mM Tris–HCl, pH 7.5, 15 mM β-mercaptoethanol, and 2 mM PMSF) to prepare soluble chromatin. Soluble chromatin (~1 mg/ml) was mixed with hydroxyapatite resin equilibrated in 50 mM sodium phosphate buffer, pH 6.8 (1.5 mg DNA/g hydroxyapatite). The volume was then increased to 10 times (10 ml/g resin) of the initial volume with the same buffer, and 5.0 M NaCl was added to the final concentration of 0.6 M. The chromatin mixed hydroxyapatite was washed 6–8 times with a large volume of 50 mM phosphate buffer containing 0.6 M NaCl. Total histones were then eluted with phosphate buffer containing 2.0 M NaCl. Eluted histones were desalted by dialysis against 10 mM Tris–HCl (pH 7.5) overnight with 3–4 changes, and precipitated with 3.5 volumes of chilled acetone. The precipitated histones were collected by centrifugation at 10,000 rpm and washed 2–3 times with chilled acetone. The pellet was air dried and dissolved in 10 mM Tris–HCl (pH 7.5). For extraction of histones by H_2SO_4, washed nuclei were suspended in 10 mM Tris–HCl (pH 7.5), 0.2 mM PMSF, and 15 mM β-mercaptoethanol at a concentration of 0.5 mg DNA/ml. Subsequently, 2.0 N H_2SO_4 was added dropwise to a final concentration of 0.4 N and incubated for 30 min at 4 °C, followed by centrifugation at 10,000 rpm for 15 min at 4 °C to collect the supernatant. The extracted histones were then precipitated by adding 3.5 volumes of chilled acetone to the supernatant and collected by centrifugation. The histone pellet was washed 2–3 times with chilled acetone, dried at room temperature, and dissolved in 10 mM Tris–HCl (pH 7.5).

2.2. Electrophoresis of histones and western blot analysis

Histones were resolved on 18% SDS–PAGE gels, and the western blot was performed with the following standard protocol. Transfer of histones onto a nitrocellulose membrane was carried out in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 10% methanol, and 0.01% SDS) overnight at 50 mA, 4 °C using a BioRad mini wet transfer apparatus (BioRad, Hercules, CA, USA). The membranes were then blocked with blocking solution (2.5% non-fat dried milk in 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 30 min. Primary and secondary antibody incubations were carried out for 1 h using the following antibodies: General H3 (Abcam, 1791), H3K4me1 (Abcam, 8895), H3K4me2 (Abcam, 32356), H3K9me (Abcam, 8896), H3K27me2 (Abcam, 24684), H3K27ac (Abcam, 45173), H3K14ac (Abcam, 46984), H3K9Ac (Abcam, 69830), and Cathepsin L (Sigma–Aldrich). Signals were detected by electrochemiluminescence (ECL) using the Fuji gel-dock system. Horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL reagents were purchased from GE Healthcare (Waukesha, WI, USA).

2.3. Preparation of H3-specific protease extract (PE)

Chicken livers from freshly sacrificed adult chickens were washed with normal saline and stored at −86 °C. Protease extraction from chicken liver and brain was performed as previously described with minor modifications [28]. All steps were performed at 4 °C. Immediately prior to homogenization, the tissue was thawed on ice and homogenized with a motor homogenizer in Solution 1 to make 33% homogenate. The homogenate was filtered through four layers of cheese-cloth and then two volumes of Solution 2 (2.1 M sucrose, 0.2 mM EDTA, 0.2 mM EGTA, 15 mM β-mercaptoethanol, and 0.2 mM PMSF) was added. Subsequently, 10.5 ml of this homogenate was layered over a 3.5 ml cushion of Solution 2 and centrifuged at 26,000 rpm for 1 h using a Beckman ultracentrifuge. The nuclear pellet was washed with Solution 3 (0.34 M sucrose, 15 mM Tris–Cl pH 7.5, 15 mM NaCl, 60 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM β-mercaptoethanol, and 0.2 mM PMSF) and suspended at a final concentration of 0.5 mg DNA/ml in lysis buffer (10 mM Tris–HCl, pH 7.5, 100 mM KCl, 3.0 mM $MgCl_2$, 0.1 mM EDTA, 2 mM β-mercaptoethanol, and 10% glycerol). Freshly made 4.0 M ammonium sulfate (pH 7.9) solution was added dropwise to a final concentration of 0.25 M while the nuclear suspension was constantly votexed on ice for 30 min. The chromatin was pelleted by centrifugation at 15,000 rpm for 30 min. Proteins in the supernatant were precipitated by slowly adding ammonium sulpfate (0.3 g/ml) while the supernatant was stirred on ice. The precipitation was allowed to occur for 30 min. Precipitated proteins were collected by centrifugation at 15,000 rpm for

30 min. The pellet was suspended in buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 2 mM β-mercaptoethanol) and dialyzed against the same buffer for 6–7 h, followed by assay for the presence of H3 protease activity.

2.4. Partial purification of H3 protease

Nuclear-extract from chicken liver had histone H3-specific protease activity. The complete purification procedure is summarized in a flow chart (Fig. 2B). The partial purification procedure of the H3-specific protease activity involved the following steps: Preparation of H3 protease nuclear-extract, DEAE anion exchange chromatography, carboxy methyl (CM) cation exchange chromatography, and Sephacryl S-200 size exclusion chromatography. All purification steps were carried out at 4 °C. The H3 endopeptidase activity was assayed with an *in vitro* cleavage reaction as described below.

2.5. In vitro H3 cleavage assay

To test the endopeptidase activity, an *in vitro* assay system was developed. Since the histone H3 was found to be cleaved in chicken liver, nuclear extract from chicken liver was used as a source of the protease and brain was used as a source of histone substrates. Histone preparations from chicken brain had intact histone H3. Hence, it was used as a substrate for the histone H3-specific protease activity. For the *in vitro* proteolytic assay, 0.5 μg of partially purified protease/nuclear extract was mixed with 5.0 μg of chicken brain core histones in 20 μl reaction volume in buffer (25 mM Tris–Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol, and 0.1 mM EDTA) and incubated at 37 °C for 1 h. The reaction was stopped by boiling in SDS–PAGE sample loading buffer. Cleavage of H3 was monitored by resolving the reaction mixture on 18% SDS–PAGE followed by Coomassie blue staining.

3. Results and discussion

3.1. The N-terminal tail of Histone H3 is cleaved during aging

Total histones from the livers of young and old chickens were isolated from the purified nuclei. Histones were then analyzed by 18% SDS–PAGE. We found the presence of one extra band that migrated between histone H2A and H4 in the histone samples

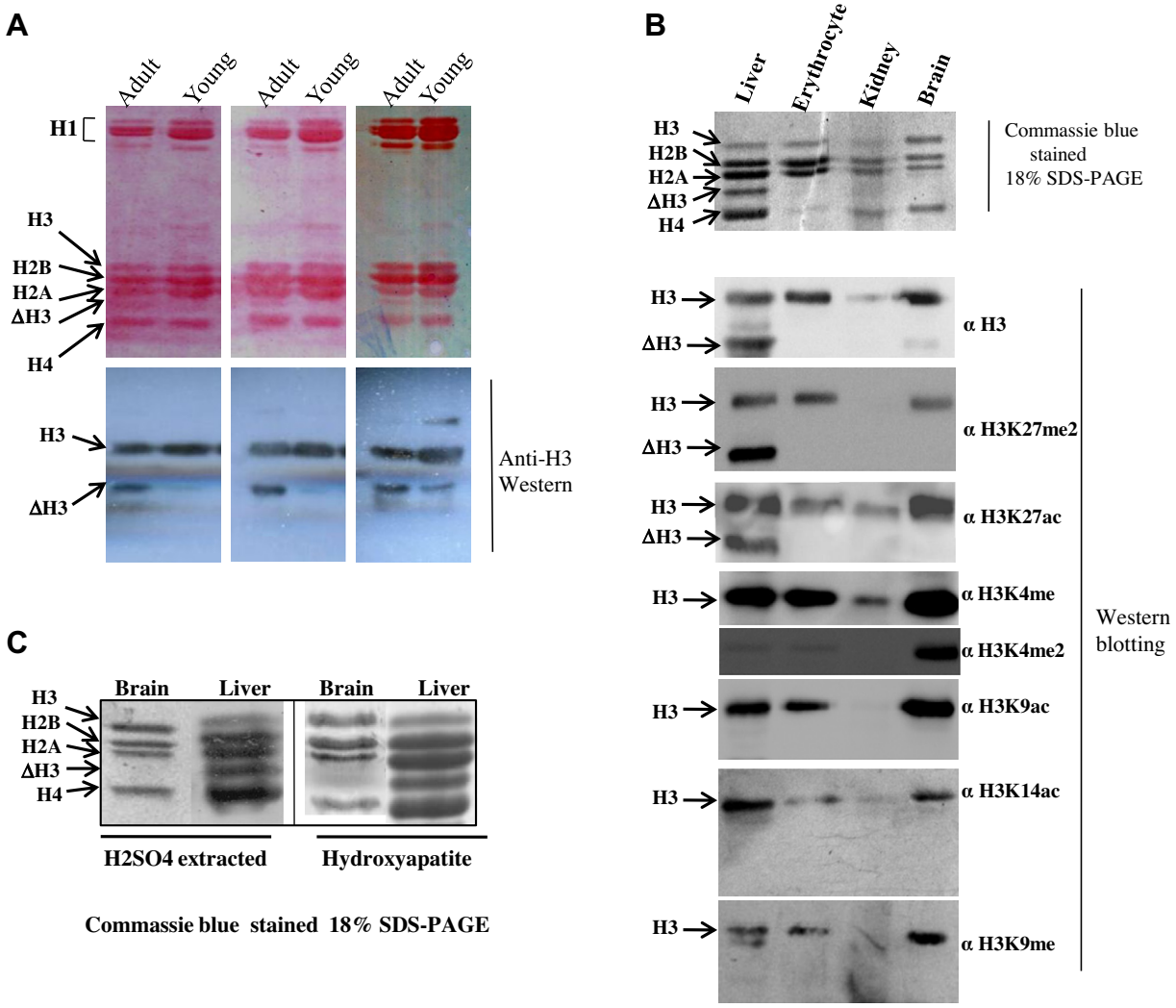


Fig. 1. N-terminal tail of histone H3 is proteolytically processed during aging. (A) Total histones from the livers of two-day-old and two-month-old chickens were isolated by H_2SO_4 extraction from nuclei that was purified through a 2.1 M sucrose cushion. Histones were analyzed by 18% SDS–PAGE followed by western blot with an anti-H3 antibody. (B) Histones were isolated from liver, brain, kidney, and erythrocytes by H_2SO_4 from purified nuclei and analyzed on 18% SDS–PAGE followed by western blot. (C) Extraction of histones from liver and brain was carried out using H_2SO_4 and hydroxyapatite methods. Antibodies specific to residues towards the N-terminal end of histone H3 (anti-H3K4, K9, and K14) do not recognize ΔH3.

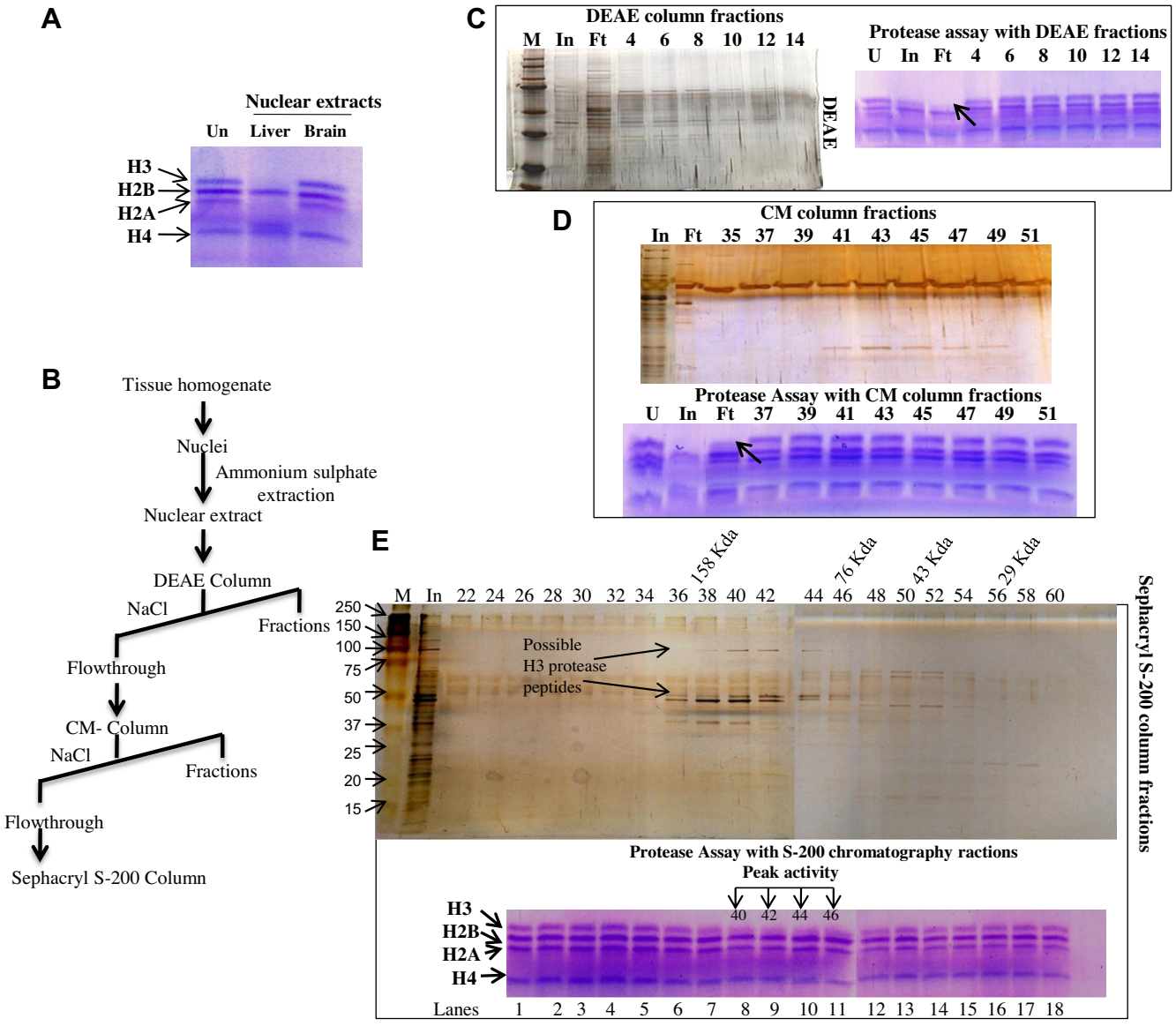


Fig. 2. Identification and partial purification of H3 protease (CLH3p) activity from liver. (A) Only liver nuclear extracts show histone H3 protease activity. Nuclei from brain and liver tissues were generated through a 2.1 M sucrose cushion. Nuclear extracts were made from brain and liver nuclei. The presence of endopeptidase activity was assayed *in vitro* by incubating nuclear extracts with the purified core histones. (B) Scheme for purification of the protease from liver nuclear extracts. (C) DEAE anion exchange column chromatography of the liver nuclear extracts. (D) Carboxy methyl (CM) cation exchange chromatography of the active fraction from the DEAE column. (E) Sephacryl S-200 size exclusion chromatography of the active fraction from the CM column. Fractions of the DEAE, CM, and S-200 were tested *in vitro* for the presence of histone H3 protease activity. Fractions of S-200 were analyzed on 10% SDS–PAGE. The arrows in all of the panels, with the exception of 'E' in the upper gel, indicate the H3 protease activity. Two arrows in the 'E' upper gel indicate the co-elution of these two peptide bands with the H3 protease activity. Molecular weight markers are shown at the top of the S-200 column. The arrows in the silver stained gel picture of S-200 fractions (panel E) indicates possible H3 protease. The peak H3 protease activity is shown in fractions from 40–46. "Un" undigested and "Ft" flowthrough.

isolated from adult liver, which is labeled as ΔH3 (Fig. 1A–C). Importantly, the appearance of the extra band was tissue specific, because we did not observe a similar band in the histone preparations from brain, kidney, and erythrocytes. Isolation of histones from purified nuclei was repeated using H_2SO_4 and hydroxyapatite methods to verify the reproducibility of the results (Fig. 1C). Based on a stoichiometric comparison, we postulated that the extra band running between histone H2A and H4 proteins on SDS–PAGE from the liver tissue extracts from adult chicken could be a proteolytically processed form of histone H3. Therefore, we performed a western blot using an anti-H3 antibody and found that the protein reacted with the antibody, suggesting that it was a processed/truncated form of histone H3 (Fig. 1A and B). Truncation of H3 has been reported in several other organisms as well, including *Tetrahymena* [10–11] as well as BHK cells infected with FMDV [18,29]. In addition, Cathepsin L H3 protease [15] in mammals and unidentified H3 protease activity in *S. cerevisiae* [25] can also induce histone H3 cleavage. To the best of our knowledge, these results are the first to describe cleavage of histone H3 in the liver of adult chickens. We next performed western blots to map specific PTMs. Histones were isolated from purified nuclei of liver, erythrocytes, kidney, and brain of adult white Leghorn chickens and the composition was analyzed by western blot (Fig. 1B). Careful analysis of the results using the indicated antibodies specific for PTMs (Fig. 1B, lower panel) revealed a correlation between histone H3 cleavage and epigenetic markers, suggesting that such markers could be involved in the regulation of this process.

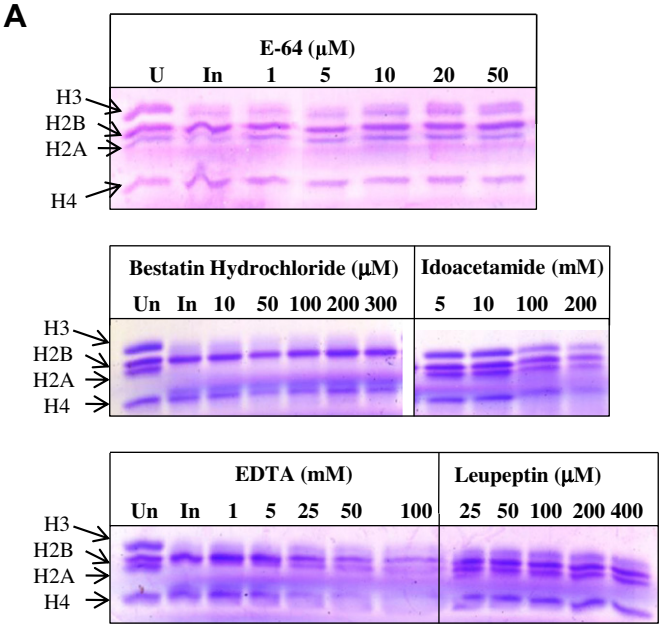
3.2. Identification of histone H3-specific protease activity in nuclei of chicken liver

Based on our finding that histone H3 was proteolytically processed in the liver of the adult chicken, we hypothesized that a protease was responsible for the cleavage. To confirm this hypothesis, we generated nuclear extracts from brain and liver tissue to assess them for the presence of H3 protease activity (Fig. 2A). In our optimized *in vitro* assay, H3 protease activity was only found in liver nuclear extracts, but not in the brain nuclear extracts. Therefore, chicken liver nuclear extracts were used as a source for the histone H3 protease activity. Nuclear extracts from chicken livers were prepared as described. Chicken brain histones were used as substrate for the histone H3 protease activity because they remained intact during the purification process and we found that they were more sensitive to protease activity than liver H3 (data not shown). Using an *in vitro* protease assay, full-length histone H3 in the mixture of histones was completely degraded when incubated with chicken liver nuclear extract (Fig. 3A). This experiment suggests that a protease activity exists in chicken liver nuclear extract, which is highly specific to histone H3, since the purified protease did not cleave any other core histone (Fig. 2E peak activity and Fig. 4A, right panel). We have named the H3 protease activity as Chicken Liver H3 protease (CLH3p).

3.3. Chicken Liver H3 protease (CLH3p) is a novel histone H3-specific endopeptidase

To characterize the H3 protease activity further, we partially purified the activity of the enzyme by employing traditional protein purification methods involving the preparation of a nuclear protease extract (PE) followed by ion exchange and gel filtration chromatography (Fig. 2C–E). We observed a few peptide bands that co-eluted with H3 protease activity in the fractions from the sephacryl S-200 gel filtration chromatography (Fig. 2E). Further characterization of these protein bands may reveal the identity of the protease responsible for this activity, which is currently ongoing. To determine the nature of the protease, we carried out proteolytic reactions using core histones and liver nuclear extracts in the presence of various protease inhibitors (Fig. 3A). *In vitro* H3 proteolysis reactions were severely inhibited in the presence of E-64, iodoacetamide, and leupeptin (Fig. 3A and B), whereas bestatin and EDTA did not inhibit the activity, suggesting that chicken histone H3 protease behaves similar to cysteine proteases. Importantly, this activity is unlike the yeast histone H3 protease, which is a serine protease. To confirm that the CLH3p activity is novel, we further compared the activity of CLH3p with the activity of a lysosomal human Cathepsin L, which has recently been reported to be a histone H3 protease. Interestingly, in our *in vitro* assays, all of the core his-

A



B

Inhibitors	Specificity	Concentration	Inhibition
E-64	Cysteine proteases	1μM-50μM	Yes
Iodoacetamide	Cysteine proteases	5mM-200mM	Yes
Leupeptin	Serine and Cysteine proteases	25μM-400μM	Yes
Bestatin Hydrochloride	Aminopeptidases	10μM-300μM	No
EDTA	Metalloproteases	1mM-100mM	No

Fig. 3. The chicken H3 protease is a cysteine protease. (A) Proteolytic activity of CLH3p is inhibited by cysteine protease inhibitors (E64, Iodoacetamide, and Leupeptin). Protease assays were conducted in the presence of increasing concentrations of protease inhibitors followed by SDS–PAGE as described in section 2. (B) A summary of the properties and effects of protease inhibitors on H3 endopeptidase activity (CLH3p). Un = undigested.

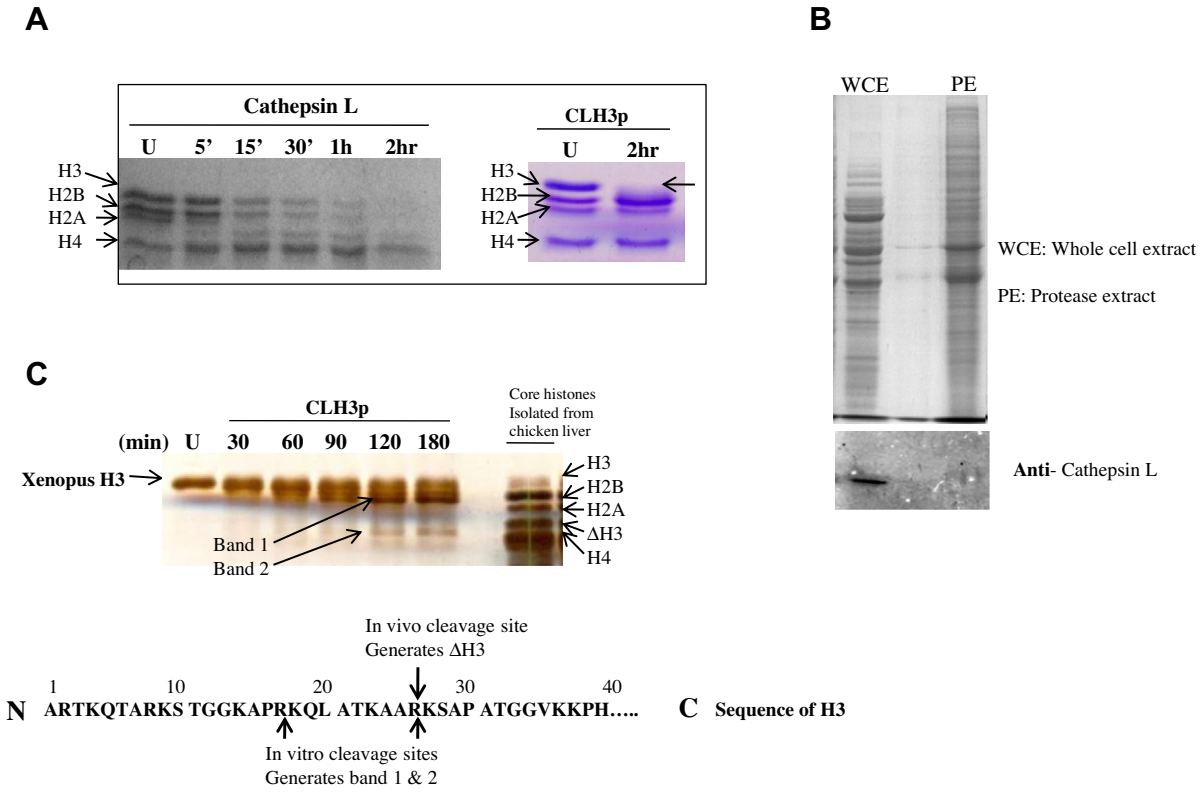


Fig. 4. Chicken Liver H3 protease (CLH3p) is a novel histone H3-specific protease that is distinct from Cathepsin L. (A) A comparison of Cathepsin L and CLH3p activity *in vitro*. Cathepsin L is non-specific and degrades all of the core histones, whereas CLH3p is highly specific for histone H3 only. (B) Whole cell extract (WCE) and protease extracts (PE) were made from liver followed by western blot with an anti-Cathepsin L antibody. (C) A proteolytic assay was conducted by incubating isolated CLH3p with the recombinant Xenopus histone H3. CLH3p generates two proteolytic products of histone H3. A molecular weight analysis of the products reveals two cleavage sites, one between Arg17 and Lys18, and the other between Arg26 and Lys27. Cleavage at the second site generates a product similar to the in vivo generated ΔH3. Un = undigested.

tones were non-specifically degraded upon incubation with recombinant human Cathepsin L, whereas CLH3p was highly specific for histone H3, suggesting that CLH3p activity is novel and specific (Fig. 4A). CLH3p does not cleave any other core histones except for histone H3. To further confirm that CLH3p was distinct from Cathepsin L, we performed a western blot of active H3 protease extract prepared from liver nuclei using the Cathepsin L antibody (Fig. 4B). We next mapped the cleavage site(s) for CLH3p within histone H3. In all of the *in vitro* protease assays, histone H3 got completely degraded and the cleaved products or intermediates were not detectable on the gel system when protease extracts were incubated with the core histones. However, there was a consistent increase in the intensity of the histone H2B protein band concomitantly with a reduction in the histone H3 band when incubated with CLH3p (Fig. 4A, right panel). To identify the cleaved product(s), we performed an *in vitro* protease reaction with recombinant H3 and CLH3p. Careful examination of a silver stained gel revealed two major bands generated by proteolytic cleavage of histone H3 upon incubation with CLH3p, suggesting that the protease may have two major cleavage sites in the protein (Fig. 4C). One major band (Band 1) was similar to the band that co-migrated with H2B, and the other band (Band 2) had a molecular weight that was similar to the *in vivo* truncated H3 (ΔH3). Based on a molecular weight comparison of the two bands with histones, the CLH3p protease probably has two cleavage sites on histone H3, with one between Arg17 and Lys18 and the other between Arg26 and Lys27. Data presented in this study suggest that the enzymatic activity of CLH3p is distinct from H3 protease activities previously reported. First, unlike Cathepsin L and the unidentified yeast enzyme, this chicken protease activity is highly specific for histone H3. Second, while Cathepsin L generates several histone H3 forms (with distinct N termini), the yeast histone H3 protease only generates a single form of histone H3 (with a cleavage site at Ala22). On the other hand, CLH3p most likely cleaves at two sites, Arg17-Lys18 and Arg26-Lys27, and is highly tissue specific. Taken together, the data presented here show N-terminal proteolytic processing of H3 and identify novel histone H3-specific protease activity that cleaves histone H3 at the N-terminus in adult chicken livers. Importantly, this protease activity may have a role in the regulation of aging and age-associated diseases.

Acknowledgments

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