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Purification and characterization of a novel histone H2A specific protease (H2Asp) from chicken liver nuclear extract

Pragnya Panda [a], Madan M. Chaturvedi [b], Amulya K. Panda [c], Mrutyunjay Suar [a], Jogeswar Satchidananda Purohit [a,b,d,*]

[a] School of Biotechnology, KIIT University, Bhubaneswar, Orissa, India
[b] Laboratory for Chromatin Biology, Department of Zoology, University of Delhi, North Campus, Delhi, 110007, India
[c] Product Development Cell, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi, 110067, India
[d] Department of Zoology, Smt. C.H.M. College, University of Mumbai, Ulhasnagar, Thane, Maharashtra, India

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ABSTRACT

The proteolysis of the N- or the C-terminal tails of histones have recently emerged as a novel form of irreversible posttranslational modifications of histones. However, there are very few reports describing purification of a histone specific protease. Here, we report a histone H2A specific protease (H2Asp) activity in the chicken liver nuclear extract. The H2Asp was purified to homogeneity and was found to be a ~10.5 kDa protein. It demonstrated high specificity to histone H2A and was an aspartic acid like protease as shown by protease inhibition assay. The H2Asp, in the *in vitro* cleavage assay generated a single clipped H2A product which comigrated along with histone H4 in the SDS-PAGE and migrated as a single band when single H2A was used as substrates. The expression of H2Asp was independent of age and was tissue specific, which was demonstrated only in the nuclear extracts of chicken liver and not from the same of other tissues like brain, muscles and erythrocytes. It was also seen that H2Asp activity also exists in other classes of vertebrates from Pisces to Mammals. This report forms the first such report describing purification of a histone H2A specific protease.

1. Introduction

Chromatin, the physiological template of the eukaryotic genome, is a dynamic polymer of DNA histone and non-histone proteins. The chromatin acts as a barrier for nuclear events like replication, transcription, repair and recombination (Taverna et al., 2007; Wang et al., 2004). One of the way by which the barrier is reversed is by posttranslational modifications of histones (Cheung et al., 2000; Wang et al., 2007). The core histones (H2A, H2B, H3 and H4) of the eukaryotic chromatin are subject to a diverse array of reversible post-translational modifications (Kouzarides, 2007; Spencer and Davie, 1999). Additionally, site-specific proteolysis of the histones has emerged as a type of irreversible post-translational modification (Allis et al., 1980). The unstructured N- or C-terminus of the histone tails are more susceptible to the proteolysis. The proteolysis has been hypothesized to have a role in histone turnover, gene regulation or removal of epigenetic marks (Jenuwein and Allis, 2001).

There are several reports depicting proteolysis of histones in correlation to physiological processes such as cellular differentiation, viral infection or aging, etc. (Duncan et al., 2008; Gonzalo, 2010; Tesar and Marquardt, 1990). During mouse embryonic stem cell differentiation, histone H3 is cleaved from the N-terminus, at multiple sites spanning between amino acids 21 and 27. The protease responsible for the cleavage is identified to be Cathepsin L (Duncan et al., 2008). It is further seen that the histone H3 clipping activity is stimulated in a specific period during the differentiation of embryonic stem cells and is regulated by covalent modification status of the histone H3. In *Tetrahymena*, 6 amino acids are proteolytically removed from the N-terminus of histone H3 in the micronuclei (Allis et al., 1980). Furthermore, the micronucleus of *Tetrahymena* also contains α, β, γ & δ H1 like forms, which are derived from proteolytic clipping of a precursor (Allis et al., 1984). On the other hand the histone H4 of the macronuclei is also clipped N-terminally during conjugation in *Tetrahymena* (Lin et al., 1991). Recently, a histone H3 endopeptidase activity has been reported in yeast by a yet to be identified protease (Santos-Rosa et al., 2009). It clips histone H3, 21 amino acids from

Abbreviations: H2Asp, Histone H2A specific protease; FMDV, Foot-and-Mouth Disease Virus; PMSF, Phenyl Methyl Sulfonate; PBS, Phosphate Buffered Saline; EDTA, Ethylenediaminetetraacetic acid; KLH, Keyhole limpet hemocyanin; EGTA, Ethylene glycol tetraacetic acid; NP40, Tergitol-type-40; DEAE, Diethylaminoethyl; GF, Gel filtration; TK, Tris KCl; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, Bovine serum albumin; MNase, Micrococcal nuclease; Asn, Asparagine; Asp, Aspartic Acid; lys, Lysin.

* Corresponding author at: Department of Zoology, Smt. C.H.M. College, University of Mumbai, Ulhasnagar, Thane, Maharashtra, India. Tel.: +91 9764036065(mobile); fax: +91 251 2731869(O).
E-mail addresses: sachin.jogesh@gmail.com, jspurohit@zoology.du.ac.in (J.S. Purohit).

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the N-terminus. The clipping is specific to the promoter regions and it precedes transcription. It is speculated that the clipping results in induction of gene expression by localized clearing of repressive signals. Cleavage of histone H3 is also observed in viral infections. In mammalian kidney cells (BHK cells) infected with Foot-and-Mouth Disease Virus (FMDV), the histone H3 is selectively degraded (Tesar and Marquardt, 1990).

Age dependent proteolysis of histone H3 is also reported in the total histones isolated from liver of Japanese quail. There was generation of a faster migrating histone H3 band in SDS PAGE with concomitant selective disappearance of histone H3 (Mahendra and Kanungo, 2000). This event is speculated to be proteolysis. Upon progesterone treatment the proteolysis can be induced even in young quail. Similar liver specific proteolysis of histone H3 is also reported from aged chicken (Mandal et al., 2012). The proteolysis is specific to liver. A histone H3 specific protease activity is also demonstrated in the chicken liver nuclear extract; however the protease is yet to be purified.

There are few reports also depicting proteolysis of histone H2A. During granulocyte differentiation, around 15 amino acids are clipped from the C-terminus of histone H2A. The chromatin domains containing the clipped H2A show lower affinity to form tetramer of core histones and is hypothesized to generate an open chromatin conformation and hence facilitate transcription (Elia and Moudrianakis, 1988). A chromatin bound proteolytic activity with unique specificity for histone H2A, is demonstrated in calf thymus nuclei (Eickbush et al., 1976). The protease is named as an H2A specific protease and has been demonstrated to remain associated to H1 containing nucleosomes. However, the protease could not be purified. From the nuclei of acute myeloid leukemia cells, two truncated forms of monoubiquitinated H2A are purified. However, the enzyme catalyzing the reactions remains obscure (Okawa et al., 2003).

To the best of our knowledge, except Cathepsin L, which is specific to histone H3, there is no report on purification of a histone specific protease till date. Here, we describe establishment of assay system for a novel histone H2A specific protease from chicken liver. We further describe purification of the Histone H2A specific protease (H2Asp) by employing a series of chromatographic steps.

2. Materials and methods

2.1. Tissues and antibodies

For the present study, white leghorn chicken was used. Tissues (liver, brain or muscle) were brought in ice from freshly sacrificed chicken in slaughter house. The tissues were immediately washed with ice cold normal saline containing 0.1 mM PMSF (Phenyl Methyl Sulfonate) and stored at −70 °C, until used. Chicken blood was collected in equal volume of ice cold PBS (Phosphate Buffered Saline), supplemented with 10 mM EDTA, 0.1 mM PMSF and 50 μg/ml $NaHSO_3$. The anti-H2A antibody and the C-terminus specific antiH2A antibodies were purchased from abcam, USA. The anti-H2A antibody was a polyclonal antibody against the full length H2A. The C-terminus specific anti-H2A antibody was raised against KLH conjugated synthetic peptide against amino acids 100–130 (C-terminal end) of H2A.

2.2. Isolation of nuclei

The nuclei from chicken tissues such as brain, blood, muscles or liver were isolated as previously described (Hewish and Burgoyne, 1973), with minor modifications (Panda et al., 2011; Panigrahi et al., 2003). All steps were performed at 4 °C ± 2 °C, unless otherwise stated. Chicken tissues were homogenized in Solution I [0.34 M Sucrose, 15 mM Tris–HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.5 mM EGTA, 2 mM EDTA, 0.5 mM Spermine, 0.15 mM Spermidine and 15 mM β-Mercaptoethanol] to make a 33% homogenate. The tissue homogenate was filtered through four layers of cheese-cloth and then was mixed with two volumes of Solution II (Solution I with 2.1 M sucrose instead of 0.34 M). 27 ml of this homogenate was layered over 9 ml cushion of solution II and centrifuged at 100,000 ×*g* for 1 hour. The nuclear pellet was washed three times in solution III (Solution I minus EDTA and EGTA). In order to isolate nuclei from erythrocytes, 0.2% NP40 was added to chicken blood and was incubated for 10 min in ice. It was centrifuged at 750 ×*g*. The pellet was washed once in solution I and twice in solution III as above.

In each nuclei isolation step the purity of the nuclei was verified under a phase contrast microscope, and the nuclei were estimated in terms of their DNA content by measuring the absorbance of an aliquot of the above nuclei at 260 nm in 5 M Urea–2 M NaCl (Sollner-Webb et al., 1976).

2.3. Isolation of histones and purification of histone H2A

Histones were extracted from the purified nuclei by acid extraction method (Hoffmann and Chalkley, 1978). Extracted histones were quantified as previously described (Sollner-Webb et al., 1976). The A_{230} ~3.3 equivalent to 1 mg histones, was taken as standard. The purity of histones was analyzed by high resolution SDS-PAGE (18%) by the method previously described (Laemmli, 1970), with minor modifications (Thomas and Kornberg, 1978).

2.4. Purification of bacterially expressed histone H2A

A recombinant *Xenopus* histone H2A, cloned in pET3a, was received as a kind gift from Dr. Tony Kouzarides, University of Cambridge, UK. The plasmid was used to transform, express and purify histone H2A from bacteria according to standard protocols (Luger et al., 1997).

2.5. Western blotting

Western blotting was carried out by standard procedures (Edmondson et al., 1996). Anti-H2A (against full length H2A) or C-terminus specific anti-H2A (against 100–130 amino acids of H2A) antibodies (Abcam, USA) were used as primary antibody in required dilutions in blocking solutions.

2.6. Preparation of chicken liver nuclear extract

Nuclear extract was prepared as previously described (Gorski et al., 1986), with minor modifications (Panda et al., 2011). Briefly, the purified nuclei from chicken liver were suspended in a final concentration of 1 mg DNA/ml in the nuclei lysis buffer (10 mM Tris–HCl pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 % glycerol, 2 mM β Mercaptoethanol). It was extracted for 30 min with 0.15 M $[NH4]_2SO_4$. It was then centrifuged at 50,000 ×*g* for 30 min at 4 ° C to pellet the chromatin. The proteins of the clear supernatant were precipitated by ammonium sulphate (0.25 g/ml). The precipitated proteins were collected by centrifugation as above and were suspended in TK buffer (25 mM Tris–HCl pH 7.5, 40 mM KCl, 0.1 mM EDTA, 10 % glycerol and 2 mM β Mercaptoethanol) and dialyzed against the same buffer overnight with three changes of the buffer. The dialyzed nuclear extract was clarified by centrifugation and the clear supernatant, hence after called as nuclear extract, was used for H2Asp assay. Protein estimation was done as described (Bradford, 1976).

2.7. H2Asp assay

A reaction condition was devised for evaluation of H2A specific protease (H2Asp) in the nuclear extract by mixing 5 μg of nuclear extract with 12 μg of chicken erythrocyte histones in a reaction mixture of 20 μl in TK buffer, and incubated at 37 ° C for 2 hours. The reaction was stopped by boiling in SDS-PAGE loading buffer and