COMPARATIVE STUDY OF DIFFERENT ACID-EXTRACTION OF HISTONES FROM SOYBEAN HYPOCOTYLS

CHU-YUNG LIN, M. W. HUANG and YIH-MING CHEN

(Received for publication Dec. 26, 1979)

Abstract: Soybean histones extracted with 67% glacial acetic acid, 0.4 N H₂SO₄, 0.25 N HCl were separated on 12.5% SDS-polyacrylamide gel by electrophoresis. The electrophoretic patterns of histones extracted with those various acids were similar, but the quantitative ratio of five classes of histones were different. Histones extracted with 0.25 N HCl or 0.4 N H₂SO₄ showed better resolution on polyacrylamide gel with less nonhistone basic proteins contamination than those extracted with glacial acetic acid. Histones extracted with glacial acetic acid showed more bands in slow moving region and these nonhistone basic proteins might be ribosomal proteins.

For identification of soybean histones, histones from peas and calf thymus were used as references. The electrophoretic pattern of soybean histones was very similar to that of pea and the electrophoretic mobilities of H3 and H4 from soybean coincided very well with those from peas and calf thymus. The order of migration for soybean histones on acetic acid-urea-polyacrylamide gel was H₄>H₃>H₂b/H₂a>H₁.

INTRODUCTION

Basic proteins called histones which are associated with DNA in eukaryotic chromatin have been isolated from a wide variety of organisms. They are separated on polyacrylamide gels into five distinct classes, which fall into three groups according to the relative contents of the basic amino acids lysine and arginine (Panyim and Chalkley, 1969; Lewin, 1975). The lysine rich histones H1 is the most variable of the histones (Kinkade et al., 1966; Rall et al., 1971; Bustin and Cole, 1969). It displays electrophoretic microheterogeneity and consists of several subfractions (Balhorn, 1971; Spiker, 1975). The number of subfractions varies from tissue to tissue in a given species, and for a given tissue, it varies from one species to another (Rall and Cole, 1971). The two classes of slightly lysine-rich histones, H2a and H2b, are intermediate in the conservation of their primary structures. The three classes of arginine-rich histones, H3 and H4, both show extensive conservation. They have primary structures that have been more highly conserved throughout the course of evolution than those of any other proteins (Spiker, 1975). Both fractions have characteristic electrophoretic mobilities even when obtained from organisms so diverse as peas and cows (Panyim et al., 1970) and the amino acid sequence show only two substitutions out of 102 amino acids in fraction H4 (Delange et al., 1969) and four substitutions out of 135 amino acids in fraction H3 (Patthy et al., 1973).

(1) This work was supported by a grant from the National Science council, Republic of China (NSC-68B–0201-02(01)).
(2) 林秋榮, Professor and Head of Botany Department, NTU, ROC.
(3) 周美慧, Teaching assistant of Botany Department, NTU, ROC.
(4) 陳益明, Professor of Botany Department, NTU, ROC.
The arrangement of histones on DNA in chromatin has been studied by electron microscopy and X-ray diffraction (Pardon et al., 1973; Oils and Oils, 1974; Miller et al., 1969). There is now considerable evidence that eukaryotic chromatin consists of a repeating subunit, termed the nucleosome, is made up of eight "inner histones" (two each of H4, H3, H2a, and H2b) associated with approximately 140 base pairs of DNA wrapped around the inner histones. The H1 class of histones is associated with the linker region (Lewin, 1975). Histones were first shown to restrict template activity in 1962 (Bonner et al., 1962). Nevertheless, several lines of evidence suggest that histones except H1 exhibit an uniformity and a consequent lack of tissue and species specificity that preclude their ability to recognize and influence particular genes. Histones are now thought to play general template restriction. Because H1 histones exhibit tissue and species specificity and also show different degree of modifications (e.g. phosphorylation, methylation, acetylation etc) in the different cell stages. Several lines of evidence suggest that modifications of H1 histones may be related to gene regulation (Turkington and Riddle, 1969; Stevely and Stocken, 1968). In spite of this, the function of H1 histones is obscure.

Because of the basic nature of histones, they can readily be extracted with either dilute hydrochloric acid (Bonner et al. 1968), or sulfuric acid (Fambrough and Bonner, 1968). In addition, 67% acetic acid extractions of ribosomal proteins (Lin et al., 1975) was used to compare with HCl or H2SO4 preparation by 12.5% SDS polyacrylamide gel electrophoresis. Pea and calf thymus histones were used as references for identification of soybean histones.

MATERIALS AND METHODS

Plant Materials

Soybean seeds (Glycine max L. CV. Kaohsiung, No. 3) were planted in moist vermiculite and germinated in the dark at 27°C. Mature hypocotyl tissues were harvested after 96 hr of germination. Solutions: Buffer A contains 30 mM MES-NaOH buffer (pH 6.0), 20 mM KCl, 10 mM MgCl2, 10 mM β-mercaptoethanol, 0.6 M sucrose, 50% glycerol. Buffer B contains 30 mM MES-NaOH buffer (pH 6.0), 20 mM MgCl2, 20 mM KCl, 10 mM β-mercaptoethanol, 30% glycerol. Buffer C contains 25 mM MES-NaOH buffer (pH 6.0), 10 mM MgCl2, 20 mM KCl, 10 mM β-mercaptoethanol, 0.6 M sucrose, 30% glycerol.

Preparation of Nuclei

The nuclei were isolated according to the method of Chen et al. (1975). 75 g of tissues were finely minced with razor blades and homogenized in 75 ml of buffer A using a Willem's polytron PCU-2 set at 4 for 30 sec. Homogenates were filtered through four layers of cheesecloth and two layers of miracloth. The filtrate was centrifuged at 3000 x g for 15 min. Nuclear pellets were scraped from underlying starch and suspended by gentle vortexing in buffer A containing 0.05% triton X-100. The suspension was centrifuged at 7700 x g for 20 min. The pellets were suspended in buffer A and centrifuged at 3000 x g for 15 min, then, the washed nuclear pellets were suspended in buffer B containing 1.0 M sucrose and layered over in buffer B containing 1.2 M sucrose. The gradient was centrifuged at 7700 x g for 15 min in a Sorvall RC-2B HB-4 Rotor. Purified nuclei were in the pellets.

Preparation of Nucleoli

Nucleoli were prepared according to Lin et al. (1975). Soybean hypocotyl tissues were finely minced with razor blades and homogenized in buffer C using a Willem's polytron PCU-2. Homogenates were filtered through four layers of cheesecloth and two layers of Miracloth, then, add triton X-100 to the filtrate and make its final concentration be 0.5%. The filtrate was homogenized again with polytron set at low speed for 2-3 min, then, the homogenates
were centrifuged at 3000 × g for 15 min. The pellets were suspended in buffer C containing 0.5% triton X-100 and homogenized with polytron set at low speed for 30 sec. The homogenates were centrifuged at 3000 × g for 10 min. This procedure was repeated twice, then, the nucleoli were purified by sucrose gradient centrifugation (as stated in nuclei preparation). Nuclei and nucleoli preparations were stained with methylene-green pyronin Y and were observed under microscope for the purity.

**Preparation of histones**

Nuclei were washed with 0.14 N and 0.35 N NaCl (centrifuged at 3000 × g for 10 min) for removal of nuclear neutral proteins. Then, histones were prepared by extraction with 0.25 N HCl, 0.4 N H₂SO₄, or 67% glacial acetic acid (Lin et al., 1975).

**Electrophoresis of Histones**

Analytical electrophoresis of soybean histones were carried out in two systems. SDS-polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (1970) as modified by Flint. Detailed experiment procedure as follows: One part of histone preparation was mixed with one part of 0.125 M tris-HCl buffer (pH 6.8), 4% SDS, 8 M urea, and 10% β-mercaptoethanol and the mixture was heated in boiling water for 2 min. Resolving gel contained 0.375 M tris-HCl (pH 8.8), 0.1% SDS, 0.05% TEMED, 12.5% acrylamide and 0.32% bis. Stocking gel contained 0.125 M tris-HCl (pH 6.8), 0.1% SDS, 0.05% TEMED, 3.5% acrylamide and 0.093% bis. Upper electrode buffer contained 0.025 M tris, 0.192 M glycine (pH 8.3), 0.1% SDS. Lower electrode buffer contained 0.025 M tris, 0.192 M glycine (pH 8.3). During electrophoresis, samples were run into stocking gels at 1 mA per gel for one hour, then, the current was increased to 2 mA per gel. Completion of the run was indicated by when the bromophenol blue dye came to 0.5 cm from the bottom of the gel. After the gels were removed from the bottom of the tube, they were fixed with 50% isopropanol and 10% trichloroacetic acid at room temperature for 1 hr. The gels were then stained with 0.1% coomassie brilliant blue in 25% isopropanol and 10% trichloroacetic acid, and destained with 10% glacial acetic acid. The gels were scanned at 600 nm with ISCO gel scanner Model 659 with absorbance monitor (model UA-4). 15% acetic acid-urea-polyacrylamide gel were prepared according to Panin and Chalkley (1969). We employed the following solutions to give a 15% polyacrylamide gel in 6.25 M urea. Solution A: 60% acrylamide (w/v) in distilled H₂O; Solution B: 43.2% of glacial acetic acid (v/v) and 4% of TEMED (w/v) in dist H₂O; Solution C: 0.2% (NH₄)₂ S₂O₃ (w/v) in 10 M urea, freshly prepared. Solution A and B were stored at 0°C and warmed to room temperature before mixing in the ratios: 1 part of B, 2 parts of A and 5 parts of C. Histones were dissolved in 0.9 N glacial acetic acid, 0.5 M β-mercaptoethanol, 8 M urea. Electrophoresis was performed in 0.9 N acetic acid at 2 mA per gel. Completion of the run was indicated by when the basic fuchsin reached to 0.5 cm from the bottom of the gel. Gels were removed from the tube and they were stained overnight with 0.1% amido black in a solution of 20% ethanol, 7% acetic acid and water. The gels were scanned at 600 nm with ISCO gel scanner Model 659 with absorbance monitor.

**RESULTS**

Because of the basic nature of histones, they can readily be extracted with dilute acids. Fig. 1a and Fig. 1b show the electrophoretic patterns of soybean histones extracted with either 67% glacial acetic acid, 0.4 N H₂SO₄, or 0.25 N HCl on 12.5% SDS-polyacrylamide gel respectively. We found that the electrophoretic patterns of histones extracted with those various acid were similar, but the ratio of five classes of histones were different. The quantitative ratio of three classes of histones (H2a & H2b & H3) to H4 is 3.8, 0.9, 1.6 for histone preparations
Fig. 1. Soybean histones extracted from nuclei preparation with 67% glacial acetic acid, 0.4 N H$_2$SO$_4$, or 0.25 N HCl were separated on 12.5% SDS-polyacrylamide gel by electrophoresis.

(a) The electrophoretic pattern of soybean histones extracted with glacial acetic acid (A), 0.4 N H$_2$SO$_4$ (B), and 0.25 N HCl (C).

(b) The gels were scanned at 600 nm with ISCO gel scanner with Model 659 with absorbance monitor (model UA-4). Soybean histones extracted with 67% glacial acetic acid (A), 0.4 N H$_2$SO$_4$ (B), 0.25 N HCl (C), and pea histones extracted with 0.25 N HCl (D) as a reference.
Fig. 2. Soybean histones extracted from nuclei preparation with 0.4 N H$_2$SO$_4$, 67% glacial acetic acid, or 0.25 N HCl were separated on 15% SDS-polyacrylamide gel and calf thymus histones were used as a reference. Soybean histones extracted with 0.4 N H$_2$SO$_4$ (A), 67% glacial acetic acid (B), or 0.25 N HCl (C), and calf thymus histones extracted with 0.25 N HCl (D) as a reference.

Fig. 3. The 15% acetic acid-urea-polyacrylamide gel pattern of soybean histones was compared to those of pea and calf thymus. Soybean histones extracted from nuclei preparation were dialyzed overnight (the dialyzed buffer solution contains 8 M urea, 0.9 N acetic acid, 0.5 M β-mercaptoethanol) and was used for 15% acetic acid-urea-polyacrylamide gel electrophoresis. The amount of proteins applied to the gel was 40-70 μg. During electrophoresis, the current was kept at 2 mA per gel.
A. soybean histones.  B. pea histones.  C. calf thymus histones.
extracted with glacial acetic acid, 0.4 N H$_2$SO$_4$, 0.25 N HCl respectively. The electrophoretic pattern of soybean histones extracted with 0.25 N HCl is most similar to that of pea (Fig. 1b). Histones extracted with glacial acetic acid showed more bands in slow moving region. Fig. 2 shows that histones extracted with 0.25 N HCl or 0.4 N H$_2$SO$_4$ showed better resolution on 15% SDS-polyacrylamide gel than that of histones extracted with glacial acetic acid. These results indicated that efficiency of various acids on histone extraction was different.

The electrophoretic pattern of soybean histones on 15% acetic acid-urea polyacrylamide gel was compared to those from peas and calf thymus (Fig. 3). Note that soybean histones extracted with 0.25 N HCl showed more bands in slow moving region compared with those from peas and calf thymus. Nevertheless, the electrophoretic pattern of soybean histones was very similar to that of pea and the electrophoretic mobility of H3 and H4 from soybean coincided very well with those from peas and calf thymus. Both histone fractions H2a and H2b from calf thymus have greater electrophoretic mobility than those from peas and soybean.

Fig. 4. The 12.5% SDS-polyacrylamide gel pattern of soybean histones was compared to that of calf thymus. Soybean histones extracted from nuclei preparation with 0.25 N HCl were dialyzed overnight (the dialyzed solution contains 0.0625 M tris-HCl buffer pH 6.8, 10 mM β-mercaptoethanol), then, one part of protein solution was mixed with one part of sample buffer containing 0.125 M tris-HCl buffer pH 6.8, 4% SDS, 8 M urea, 10% β-mercaptoethanol. The mixture was heated in boiling water for 2 min and was readily for SDS-polyacrylamide gel electrophoresis. The amount of proteins applied to the gel was 100-150 μg. During electrophoresis, samples were run into stockgels at low current (about 1 mA per gel) for about one hour, then the current was kept at 2 mA per gel until the bromophenol blue dye came to 0.5 cm from the bottom of the gel. After gels were removed from the bottom of the tube, they were fixed with 50% isopropanol, 10% TCA for 30 min and then stained with 0.1% coomasie blue and destained with 10% glacial acetic acid.

A. soybean histones.
B. calf thymus histones.
histone fraction H2a & H2b from peas and soybean couldn't be separated on SDS gel. So the order of migration for aminal histones were H4>H2a>H2b>H3>H1, but for plants were H4>H3>H2a/H2b>H1. All species have histones of low electrophoretic mobility presumably H1 histones. The electrophoretic mobility and number of H1 histones were highly variable among soybean, peas, and calf thymus. This could be due to their different primary structures and due to results of modifications in different organisms. Soybean histone fraction H1 has two subfractions designed as H1a and H1b and the electrophoretic mobility of H1a was slower than that of H1b.

The SDS-polyacrylamide gel pattern of soybean histones was compared to standards of calf thymus in Fig. 4. Note that the electrophoretic mobility of H1a in SDS gel was greater than that of H1b. These differences could be due to differences in molecular weight and in molecular charge of H1a and H1b.

Histones extracted from nuclei and nucleoli were subjected to 15% acetic acid-urea-polyacrylamide gel in Fig. 5. Note that histones extracted from nucleoli showed more bands in slow moving region. Because nucleoli were the site of ribosome assembly, these results indicated that nonhistone basic proteins may be ribosomal proteins and they will be efficiently extracted with glacial acetic acid.

Fig. 5. Soybean histones extracted from nuclei and nucleoli preparations were separated by electrophoresis on 15% acetic acid-urea-polyacrylamide gel. Three-day old soybean seedlings were treated with $2.5 \times 10^{-4} \text{M} 2,4-\text{D (pH 6.0)}$ for 24 hours, then, mature hypocotyl tissues were harvested for nuclei and nucleoli preparations (described in material and methods).

(a) The electrophoretic gel patterns of soybean histones extracted from nuclei and nucleoli on 15% acetic acid-urea-polyacrylamide gel.
A. Histones extracted from nuclei preparation.
B. Histones extracted from nucleoli preparation.

(b) The gels were scanned at 600 nm with ISCO gel scanner Model 659 with absorbance monitor (model UA-4).
A. -----: soybean histones extracted from nuclei.
B. - - - - : soybean histones extracted from nucleoli.
DISCUSSION

Approach to resolving the structure of chromatin has been developed from analysis of histone properties. The striking feature of histones is their constancy in all well defined eukaryotes: (a) the ratio of total histone to DNA is practically constant for a variety of tissues and organisms; (b) the number of histones is small, though the five major fractions exhibit heterogeneity due to chemical modifications and, in the case of the lysine-rich histones, sequence differences specific to organs and to tissues; and (c) striking conservation of sequence is found for histone H4 and H3. The constancy indicates that histones except H1 participate in essential functions of the chromosome which have been highly conserved through evolution. It is thought that the major function of histones is the conservation and control of the structure of the eukaryotic chromosome during the cell cycle and that this control is exercised through chemical modifications of histones which enable interactions between histones and DNA to be modified throughout the genome.

Due to the basic nature of histones, they could readily be extracted with either dilute hydrochloric acid or sulfuric acid. In this paper, soybean histones extracted with 67% glacial acetic acid, 0.4 N H$_2$SO$_4$, or 0.25 N HCl were compared on 12.5% SDS-polyacrylamide gel by electrophoresis. We found that the electrophoretic patterns of histones extracted with those various acids were similar, but the quantitative ratio of five classes of histones were different. Histones extracted with 0.25 N HCl or 0.4 N H$_2$SO$_4$ showed better resolution on polyacrylamide gel with less nonhistone basic proteins contamination than those extracted with glacial acetic acid. The recovery of histones extracted with glacial acetic acid was less than those extracted with 0.25 N HCl or 0.4 N H$_2$SO$_4$. Since ribosomal proteins were normally extracted with glacial acetic acid and histones extracted from nucleoli (site of ribosome assembly) preparation showed more bands on polyacrylamide gel than those extracted from nuclei preparation, this results indicated that nonhistone basic proteins which could be separated on SDS-polyacrylamide gel in slow moving region could be ribosomal proteins and they would be efficiently extracted with glacial acetic acid.

Peas and calf thymus histones were used as references for soybean histones identification. We found that soybean histones showed more bands in slow moving region compared with those from peas and calf thymus. These nonhistone basic proteins could be ribosomal proteins and were difficultly eliminated from soybean histone preparation. The electrophoretic pattern of soybean histones was very similar to that of peas and the electrophoretic mobilities of H3 and H4 from soybean coincided very well with those from peas and calf thymus. As is now well known, pea H4 and H3 sequence are nearly identical to those of calf thymus (Elgin and Weintraub, 1975), and a wide variety of land plants possesses histones of the same electrophoretic mobilities as calf and pea H3 and H4. (Spiker, 1975). The highly conserved sequence of histone H3 and H4 implies that each and every residue along the polypeptide chain is essential for the function of the histone, and that this function is identical in all eukaryotes. The electrophoretic mobility and number of H1 histones was highly variable in the species investigated. This variation may be due to the different primary structure and specific modifications of H1 histones. There was evidence that dividing cells showed extensive phosphorylation induced electrophoretic microheterogeneity and that non-dividing cells had essentially no phosphorylated lysine-rich histone species (Ballhorn et al., 1971). These H1 difference have already been used to investigate gene expression in hybrid corn (Stout and Phillips, 1973) and may well be used in the future to study phylogenetic relationships between closely related taxa. The remaining two plant histone fractions H2a and H2b are intermediate in the conservation of their primary structure. In both acetic acid-urea and SDS-polyacrylamide gels, plant histone fractions H2a and H2b showed lower electrophoretic mobilities than that of
histone fraction H3, whereas, in animals, H2a and H2b migrate more rapidly than H3. The role of histone fractions H2a and H2b in chromatin structure is still under investigation. However, there was evidence that the chromatin from fungi (Lohr and Van Holde, 1975), Protista (Gorovsky and Keevert, 1975), and plants (Mghee and Engel, 1975) were organized similarly to that of animals. Therefore, it is likely that organisms from these three kingdoms will have histones which are the functional equivalents of animal H2a and H2b.

So, the order of migration for soybean histones is H4>H3>H2a/H2b>H1. Due to the contamination of nonhistone basic proteins in histone preparation, further purification and study by two dimensional gel electrophoresis are necessary.

REFERENCES


