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CHARACTERIZATION OF CHROMATIN FROM *TRITICUM AESTIVUM*

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Abstract—Chromatin was isolated from wheat seedlings using chromatin aggregation in the presence of cations and dispersion in the absence of these cations. The preparation contained some contaminating RNA. The protein, DNA and RNA moieties were separated from each other by ion exchange chromatography on hydroxyapatite in the presence of 5 M urea and 2 M NaCl. The histone and non-histone proteins were subsequently separated on CM-cellulose and the histones partially separated from one another. The various protein fractions were characterized by urea–acetic acid and sodium dodecyl sulphate polyacrylamide gel electrophoresis.

INTRODUCTION

THE BEHAVIOUR of eucaryotic chromosomes is undoubtedly controlled to a considerable extent by chromosomal and chromosome-associated proteins. However, isolation and characterization of these proteins has not been easy because of their interactions with each other and with nucleic acids, and because of difficulties of assay.

Many methods of chromatin fractionation have relied on the use of drastic conditions such as acid (for histone proteins), alkali (for non-histone proteins), organic solvent and ionic detergent extractions, all of which are likely to cause denaturation and loss of *in vivo* biological activity.[1–3] In addition, many of the chromatin fractionation procedures have been orientated towards only certain kinds of components, the rest being neglected. Because of such inadequacies, several laboratories[4–7] have recently attempted to overcome these difficulties and develop techniques of fractionation which are designed to retain the biological properties of the component molecules.

In this laboratory, we are especially interested in the structure and function of plant chromosomes and were in need of some simple methods for fractionating chromatin and chromatin-associated proteins. The following criteria seemed desirable. (1) Extensive purification of chromatin should be avoided if chromatin-associated activities are not to be lost.

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[1] ELGIN, S. C. R. and BONNER, J. (1970) *Biochemistry* **9**, 4440.
[2] BENJAMIN, W. and GELLHORN, A. (1968) *Proc. Nat. Acad. Sci. U.S.A.* **59**, 262.
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[5] GRAZIANO, S. L. and HUANG, R. C. (1971) *Biochemistry* **10**, 4770.
[6] MACGILLIVRAY, A. J., CAMERON, A., KRAUZE, R. J., RICKWOOD, D. and PAUL, J. (1972) *Biochim. Biophys. Acta* **277**, 384.
[7] VAN DEN BROEK, H. W. J., NOODEN, D., SANDERS SEVALL, J. and BONNER, J. (1973) *Biochemistry* **12**, 229.

(2) The fractionation procedures should aim to conserve the biological activities of the components and therefore should avoid the use of alkalis, acids, organic acids and detergents. (3) The fractionation procedure should not result in the loss of components. (4) The procedures should be suitable for both analytical as well as preparative analyses.

In this report we describe the isolation, fractionation and characterization of a chromatin fraction from wheat which was developed with these criteria in mind.

RESULTS AND DISCUSSION

Isolation of chromatin

For the rapid isolation of a crude chromatin fraction from wheat seedlings we have used cations to condense the chromatin.[8] In the presence of K^+, Ca^{2+} or Mg^{2+} in a sucrose–TKMC extraction buffer,[9] more than 80% of the extracted DNA could be repeatedly sedimented at 4000 *g*. The crude 4000 *g* pellet of the filtered cell homogenate contained some intact nuclei, in addition to the condensed chromatin and was heavily contaminated with starch grains, and nuclear, proplastid and cell wall debris. After three or four washes in sucrose–TKMC buffer,[9] two successive homogenizations of the 4000 *g* pellet in 0·01 M Tris–HCl buffer, pH 8·0 lacking K^+, Ca^{2+} and Mg^{2+} led to the dispersion of more than 80% of the DNA such that it could not be sedimented at 15000 *g*. The control of chromatin dispersion by the concentration of one ion, K^+, is shown in Fig. 1. A single homogenization of the crude 4000 *g* pellet in 0·01 M Tris–HCl buffer, pH 8·0 led to 75% of the DNA not being sedimented at 15000 *g* while following homogenization in the presence of 100 mM KCl only 28% could not be sedimented at 15000 *g*.

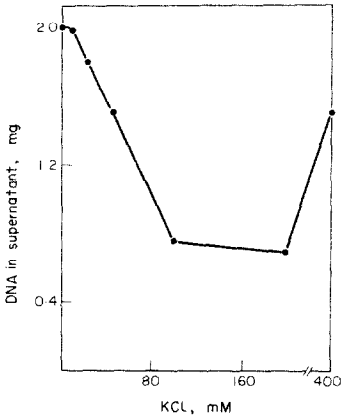

FIG. 1. EFFECT OF K^+ ON CONDENSATION OF CHROMATIN.
Equal amounts of a washed, crude chromatin 4000 *g* pellet were homogenized in aliquots of 0·25 M sucrose 0·01 M Tris–HCl buffer pH 8 containing different concentrations of KCl and centrifuged at 15000 *g* for 15 min. The amount of DNA in each supernatant was estimated chemically. *ca* 0·67 mg remained in the 15000 *g* pellet after homogenization in the absence of KCl.

The increase in dispersed chromatin at concentrations above 200 mM KCl was almost certainly due to the release of proteins from chromatin with resultant dispersion.[8] Homogenization of the crude, washed 4000 *g* pellet in 0·01 M Tris–HCl buffer, pH 8·0 containing 2 M NaCl, which also releases proteins from chromatin,[4–7] did not solubilize significantly

[8] LEAKE, R. E., TRENCH, M. E. and BARRY, J. M. (1972) *Exp. Cell Res.* **71**, 17.
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more chromatin, protein and DNA than in the absence of NaCl but it did solubilize considerably more RNA, most probably from the nucleoli.

The 15000 *g* supernatants had characteristic spectra of chromatin with an absorption peak at 260 nm and a minimum at 240 nm, a 260/240 ratio of *ca* 1·45–1·8, a protein/DNA ratio of between 1·4 and 1·8 and a 260/310 nm ratio always greater than 20.[10] Thus, this relatively simple procedure extracted more than 80% of the condensed chromatin in a relatively pure form, as indicated by its UV spectrum and protein/DNA ratio,[10] but it had a DNA/RNA ratio of *ca* 4. Whether this chromatin is representative of the 20% non-extracted chromatin is not known. The non-extracted chromatin could be firmly attached to nuclear membranes, nucleoli or maintained in a condensed configuration by proteins or other macromolecules. The proteins isolated by the NaCl treatment are qualitatively, at least, similar to the proteins isolated by removal of cations, as judged by urea–acetic polyacrylamide electrophoresis.

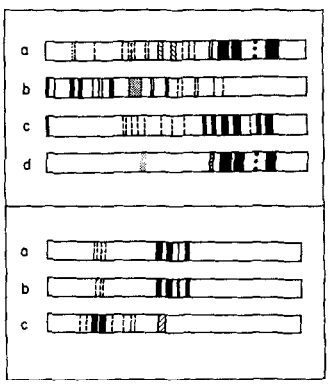

FIG. 2. ACETIC ACID–UREA POLYACRYLAMIDE GEL ELECTROPHORESIS OF CHROMATIN PROTEINS.
(a) Fraction A from hydroxyapatite column. (b) Fraction B from hydroxyapatite column. (c) Proteins extracted from washed crude chromatin 4000 *g* pellet by homogenization in sucrose–TKMC buffer containing 0·4 N H_2SO_4. (d) Proteins from fraction A retained by CM-cellulose at 0·225 M Na^+ pH 6·9 in the presence of 5 M urea. Electrophoresis was from the anode (left) to cathode (right) for 2 hr at 130 V (*room temp.*).

FIG. 3. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF CHROMATIN PROTEINS
(a) Fraction A from hydroxyapatite column. (b) Proteins from fraction A retained by CM-cellulose at 0·225 M Na^+ pH 6·9 in the presence of 5 M urea. (c) Fraction B from hydroxyapatite column. Electrophoresis was from the cathode (left) to the anode (right) for 3 hr at 5 mA/gel (room temp.). The bromophenol blue marker dye moved slightly ahead of the fastest moving proteins.

Fractionation of chromatin

Essentially all chromatin protein can be removed from DNA by a combination of 2 M NaCl and 5–7 M urea,[4,6] Our chromatin preparations were therefore made up to 2 M NaCl and 5 M urea by addition of the two solids and dialysed at 3° for 16 hr against 2 M NaCl, 5 M urea, 2 mM phosphate buffer, pH 6·8, containing 0·1% mercaptoethanol. Mercaptoethanol was added to reduce the binding of histones to non-histones.[4] Separation of the protein and RNA components from the DNA has been achieved in different laboratories by centrifugation,[4] gel chromatography,[5,7] or ion-exchange chromatography.[6,11]

[10] BONNER, J., CHALKLEY, G. R., DAHMUS, M., FAMBROUGH, D., FUJIMURA, F., HUANG, R. C., HUBERMAN, J., JENSEN, R., MARUSHIGE, K., OHLENBUSCH, H., OLIVERA, B. and WIDHOLM, J. (1968) *Methods in Enzymol*, Vol. 12B, p. 3, Academic Press, New York.

[11] MACGILLIVRAY, A. J., CARROLL, D. and PAUL, J. (1971) *FEBS Letters* **13**, 204.

We chose ion-exchange chromatography on hydroxyapatite as detailed by MacGillivray *et al.*[6,11] since in one step this allowed a partial separation of histones, non-histones and RNA from each other and complete separation from DNA, using chromatin from several animal tissues. The dialysed chromatin sample in 2 M NaCl, 5 M urea, 2 mM phosphate buffer, containing 0·1% mercaptoethanol, pH 6·8, was applied to a hydroxyapatite column equilibrated with 5 M urea, 2 M NaCl, in 2 mM phosphate buffer and eluted.

One group of proteins did not adhere to the hydroxyapatite while essentially all the remaining protein and some RNA eluted in buffer containing 85 mM phosphate. Most of the RNA eluted at phosphate concentrations between 85 mM and 200 mM and the DNA, essentially free of protein, could be eluted with 0·5 M phosphate. The 260/230 ratio of the first protein peak in experiments where mercaptoethanol was omitted from the sample dialysis medium (mercaptoethanol has high absorption in UV) was 0·25 which is more characteristic of histones which lack tryptophan. The 260/230 nm ratio of the second protein peak was 0·8.

Analysis of the two protein fractions by urea–acetic acid[5] polyacrylamide gel electrophoresis, after extensive dialysis and lyophilization, indicated that the fraction which did not adhere to the hydroxyapatite in 2 mM phosphate (fraction A) contained at least 20 proteins (Fig. 2). The five fastest moving bands in the urea-acetic acid system accounted for about 60–70% of the protein stained in the gel and had a migration pattern characteristic of histones.[4–6] In sodium dodecyl sulphate (SDS) gels[12] four faster-moving bands characteristic of histones (MW *ca* 10–20000 daltons) accounted for *ca* 90% of the stain in the gel (Fig. 3a). Four minor bands representing much higher MW proteins were also present. No slow moving bands in the relative quantities of the major slower moving bands in the urea–acetic acid gels were observed on SDS gels indicating that these latter proteins are probably acidic and of fairly low MW.

Conclusive analyses, by urea–acetic acid gel electrophoresis, of the proteins eluted from hydroxyapatite with 85 mM phosphate (fraction B) were prevented by the low solubility of these proteins in 2·5 M urea, 0·9 N acetic acid with 0·1% mercaptoethanol. Non-histone chromatin proteins are characteristically relatively insoluble.[4–6] Of the proteins that did enter the urea–acetic acid gels only a small proportion of them migrated as fast as most of the protein in fraction A (Fig. 2b). SDS gel electrophoresis of fraction B proteins indicated that most were of considerably higher MW than the major proteins of fraction A (Fig. 3c).

The behaviour of the chromatin components on hydroxyapatite was similar to that described by MacGillivray *et al.*[6,11] From comparisons of their results with those reported here, it can be concluded with certainty that fraction A consists of histones with a small contaminating proportion of more acidic non-histone proteins, while the proteins of fraction B are generally more acidic and of higher MW than histones.

Good separation of histones and non-histone proteins has been reported using the strong cation exchanger SP-Sephadex in the presence of 0·23 M NaCl and 7 M urea,[5] and on various weak cation exchangers in the presence of guanidinium chloride, formic or hydrochloric acids and urea.[10] The SP-Sephadex gel suffers considerable shrinkage over the changes in salt concentration required for histone fractionation, making shallow gradients difficult to maintain, while the use of guanidinium chloride makes UV monitoring of column effluents difficult and expensive and guanidinium has to be removed before the protein can be precipitated with TCA.[10] We therefore chose carboxymethyl cellulose chro-

[12] WEBER, K. and OSBORN, M. (1969) *J. Biol. Chem.* **244**, 4406.

matography in the presence of 5–7 M urea, using Na^+ as the counter ion for the separation of basic and acidic proteins and for a partial separation of the basic proteins. Figure 4 shows the elution pattern of the protein fraction A applied, after dialysis against 5 M urea 50 mM sodium phosphate buffer pH 6·9, to the CM-cellulose column equilibrated with the dialysis buffer. The fraction not adhering to carboxymethyl cellulose at 75 mM Na^+ was found to be of low MW proteins. At 175 mM and *ca* 225 mM Na^+ small amounts of proteins eluted. The gradient of 225–475 mM Na+ produced a partial separation of basic proteins into at least 3 peaks. The remaining very basic protein fraction could be eluted with a Na^+ conc. of above 0·8 M. The elution profile is very similar to that reported for the fractionation of histones from Chick brain chromatin on SP-Sephadex at pH 5·2.[5] Although this fractionation is incomplete it has proved sufficient for distinguishing differential rates of synthesis of different histones in certain plant tissues (unpublished results).

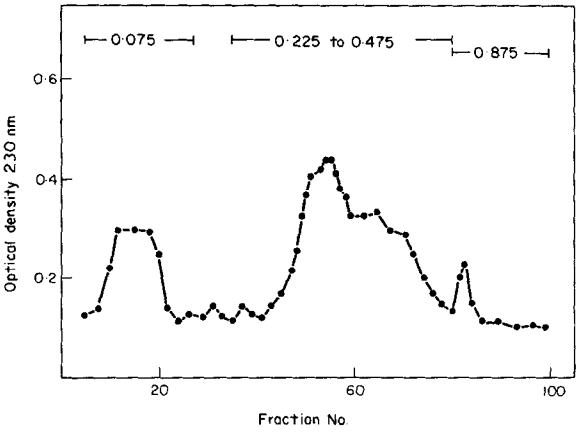

FIG. 4. SEPARATION OF NON-HISTONE AND HISTONE PROTEINS ON CM CELLULOSE.
Fraction A from the hydroxyapatite column was dialysed against 5 M urea 50 mM sodium phosphate buffer pH 6·9 and applied to a CM-cellulose column equilibrated with 5 M urea 50 mM sodium phosphate buffer pH 6·9. After washing the column with 5 M urea 50 mM sodium phosphate buffer containing 100 mM NaCl, a linear gradient of 5 M urea 50 mM sodium phosphate buffer containing NaCl from 150 to 400 mM followed by 5 M urea 50 mM sodium phosphate buffer containing 0·8 M NaCl were used to elute the protein from the column. The Na^+ concentrations over the appropriate parts of the elution profile are shown at the top.

Analyses by urea–acetic acid gel electrophoresis of the proteins retained to the CM-cellulose column have shown that more than 90% of the protein can be resolved into 4 or 5 fast moving bands characteristic of histones but in overloaded gels some slower-moving proteins also become apparent (Fig. 2d). To investigate whether these proteins present in only very small proportions, were acidic non-histone contaminants or non-histone basic proteins from the crude chromatin fraction, the basic proteins from the chromatin fraction were extracted with 0·4 N H_2SO_4 in sucrose–TKMC buffer and analysed by urea–acetic acid gel electrophoresis. In addition to proteins migrating at a similar rate to the histones, some acid–soluble (basic) proteins, present only in minor quantities, did move much more slowly (Fig. 2c). Thus the minor proteins adhering to the CM-cellulose could be high MW basic proteins. The presence of some contaminating high MW proteins in the fraction retained by the CM-cellulose was confirmed by SDS electrophoresis (Fig. 3b). Several authors have found contamination of purified histone fractions with high MW acidic proteins.[4,5]

Chromatography of fraction B proteins on SP-Sephadex in 0·23 M NaCl and 7 M urea in 10 mM acetate buffer pH 5·2, under which conditions histones do bind to the gel while non-histones do not, failed to demonstrate that the fraction contained any significant proportion of basic histone proteins. This is in agreement with the electrophoretic results and the results of MacGillivray *et al.*[6]

The aggregation and insolubility properties of the non-histone chromatin proteins make fractionation of them difficult. We attempted to separate them on DEAE-cellulose at pH 8·0 in the presence of 3 M urea as described for rabbit non-histone proteins by Levy, Simpson and Sober.[4] With a linear NaCl gradient the separation is poor and the proteins cannot be well separated from the contaminating RNA. The non-histone protein from rabbit chromatin could be separated from the contaminating RNA on DEAE-cellulose.[4] For good separation of non-histone proteins in wheat other methods will have to be used. However, even the degree of fractionation of wheat non-histone protein obtained here has enabled us to distinguish the preferential synthesis of certain chromatin-associated acidic proteins in cereal anthers (unpublished results).

EXPERIMENTAL

DNA and protein estimations. DNA was estimated by its absorption at 260 nm and by the diphenylamine test using calf thymus DNA as a standard.[13] Protein was estimated chemically by the procedure of Lowry *et al.*[14] using bovine serum albumin (fraction V) as a standard. Protein and nucleic acid in column effluents were estimated by absorption at 230 and 260 nm respectively. The pH given are pH at room temp.

Growth of seedlings. Seeds of hexaploid wheat *Triticum aestivum* were incubated under moist conditions at 3° for several days and then germinated at 25° before being planted in a sterilized soil-less compost in trays. After growth in the dark at room temp. or in an incubator at 20 ± 1° for about 7 days, the aerial parts of the seedlings were harvested, wrapped in al-foil and stored at −20°.

Isolation of crude chromatin. The frozen seedling tissue was homogenized at 3° in a blender for *ca* 30 sec. at maximum speed in 4× its wt of 50 mM Tris–HCl buffer pH 8 containing 25 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$ and 0·25 M sucrose[9] (sucrose–TKMC buffer). The homogenate was squeezed through a 25 μm nylon mesh or through several layers of muslin and the debris re-extracted in the blender in a small vol. of sucrose–TKMC buffer. The combined filtrates were centrifuged at 4000 *g* for 10 min. The pellet was subsequently washed 3–4× by homogenization in sucrose–TKMC buffer and centrifugation at 4000 *g* for 10 min. The remaining pellet was then homogenized in a small vol. of 10 mM Tris–HCl buffer pH 8 and centrifuged at 15000 *g* for 15 min. and subsequently re-extracted by a second homogenization and centrifugation in 10 mM Tris–HCl buffer. The combined supernatants constituted the crude chromatin extract. Homogenization of all pellets after centrifugation was performed by hand in a glass homogenizer with a close-fitting Teflon pestle.

Ion exchange chromatography. Fractionation of chromatin on hydroxyapatite was as described by MacGillivray *et al.*[6,11] and as indicated in Results. Chromatography on SP-Sephadex, CM cellulose and DEAE-cellulose was by conventional column chromatography techniques using the buffer systems indicated in Results.

Polyacrylamide gel electrophoresis. Protein samples for electrophoresis were dialysed for 3 days against 50 mM HOAc and then lyophilized to dryness. For urea–acetic acid polyacrylamide gel electrophoresis, the lyophilized protein samples were dissolved in 2·5 M urea, 0·9 M HOAc and 0·1% mercaptoethanol by incubation at 37° for 2–4 hr. Electrophoresis was exactly as described by Graziano and Huang.[5] For MW analyses by electrophoresis in SDS the lyophilized proteins were dissolved in 1% SDS 0·1% mercaptoethanol 0·01 M phosphate buffer pH 7 by incubation at 37° for 2 hr and separated by electrophoresis as described by Weber and Osborn[12] except the gel and buffers were 50 mM phosphate buffer, pH 7. All gels were stained for 1 hr with 0·25% Coomassie blue in 5% MeOH 7% HOAc and destained in 7% HOAc for 2–3 days on a rotary shaker at 25°.

[13] BURTON, K. (1956) *Biochemistry* **62**, 315.
[14] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.