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ISOLATION AND ELECTROPHORESIS OF NUCLEAR PROTEINS OF BEAN*

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Abstract—Methods are described for the fractionation of the acidic and basic proteins associated with the chromatin of plant tissue. Polyacrylamide gel systems are also described which give optimal electrophoretic resolution of these protein fractions. Utilizing these techniques, comparative studies revealed a differential specificity of these proteins for the undifferentiated hook and the more differentiated lower hypocotyl tissue of bean hypocotyls. The acidic nuclear protein fractions exhibited a difference in gel patterns of the two stages of development while the histones showed little difference. The ratio of the ethanol–HCl extracted histones to the HCl extracted histones appeared to increase with development.

INTRODUCTION

INTEREST in proteins of the cell nucleus has increased in recent years. These proteins have been classified into two general categories: (1) the basic histones which are associated with the deoxyribonucleic acids (DNA), and (2) the acidic proteins which comprise about 70 per cent of the total proteins of nuclei and are associated with the nucleoli, DNA, and sap of nuclei.

Attempts have been made to find out whether nuclear proteins possess tissue or species specificity. Studies of histones have led to conflicting results.[1] Recent investigations have shown only a small, if any, tissue or species specificity of histones in adult organisms.[2–5] Qualitative differences in histones have been observed in some instances in extremely specialized cells or in comparisons of embryonic and adult tissues.[6–18]

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Studies of acidic nuclear proteins and their specificity to tissues have been few. Tumor cells have been shown to differ from normal cells in acidic nuclear proteins.[19] We learned that some acidic nuclear proteins are specific to plant tissues of different stages of development.[20]

Thus it would seem that further studies of nuclear proteins as related to differentiation and development would be informative to the plant biologist.

The majority of techniques of isolation and electrophoresis of nuclear proteins have been worked out for animal tissue. Thus, we found it necessary to adapt existing techniques for use with plant tissues. In this paper we describe techniques of isolation and polyacrylamide gel disc electrophoresis of acidic and basic nuclear protein fractions from plant tissue of different stages of development.

RESULTS AND DISCUSSION

Protein Extraction and Purification

The acidic ribonuclear and acidic chromosomal protein fractions initially contained a high amount of nucleic acids. Precipitating these proteins by lowering the pH to 5·0 eliminated most of the nucleic acids; however, a small portion of the nucleic acids remained in both fractions even after a series of precipitations. Quantitative studies with the Lowry method[21] and qualitative studies using gel electrophoresis, showed only a minor loss of protein, and no pattern changes due to the precipitation methods.

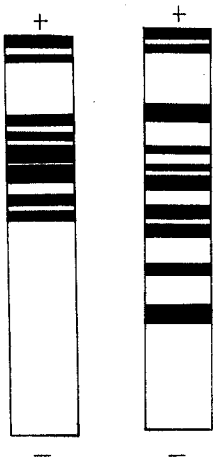

FIG. 1. TOTAL HISTONE FROM THE CHROMATIN OF HOOK (left) AND LOWER HYPOCOTYL (right). The gels were 15%, pH 2·9. Top: anode; bottom: cathode. 300 μg protein/column.

The acetone precipitations of the histones were also quantitatively complete. Total precipitation of the proteins in the ethanol–HCl fraction could only be achieved by a combination of acetone and pH adjustment to 10. These procedures caused some variation in the electrophoretic patterns as can be seen by comparing Fig. 1 with Fig. 2 (cols. 5 and 6).

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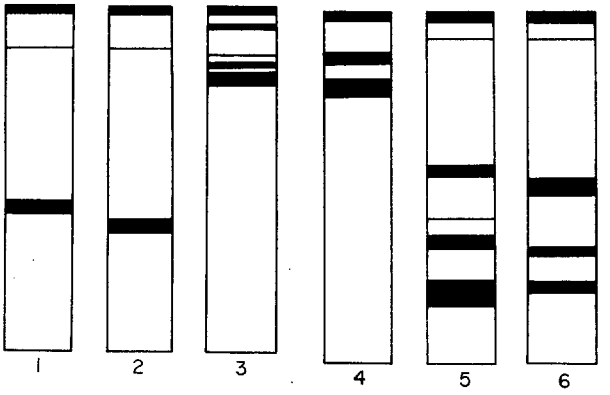

FIG. 2. SUBFRACTIONATED HISTONE FROM CHROMATIN. HCl HISTONE FROM LOWER HYPOCOTYL (col. 1) AND HOOK (col. 2); ETHANOL–HCl HISTONES FROM LOWER HYPOCOTYL (col. 3) AND HOOK (col. 4); TOTAL HISTONES FROM LOWER HYPOCOTYL (col. 5) AND HOOK (col. 6).
The gels are 15%, pH 2·9. Top: anode; bottom: cathode. Columns 1, 2, 3, and 4: 200 μg protein/column; columns 5 and 6: 300 μg protein/column.

Polyacrylamide Gel Electrophoresis

The 15 per cent gel systems used (see Experimental) gave the best results as compared with other gel systems studied, i.e. those with spacer gels and of 7 per cent acrylamide concentrations. The spacer gels used in most standard polyacrylamide gel systems (a 2–4 per cent acrylamide concentration, pH 6·7) prevented the acidic proteins from entering the separating gel. It is likely that it is a pH effect which is preventing the acidic proteins from entering the separating gel. The 7 per cent acrylamide separating gel did not give fixed, consistent patterns for acidic proteins, but only diffusely staining areas. This indicated that the gel pores were not small enough to separate the proteins or to maintain sharp bands before they were fixed by the stain. The 15 per cent gel systems gave the best patterns for both the histones and acidic proteins as is shown in the figures.

Nuclear Proteins and Development

Histones. The ratio of the ethanol–HCl extract to the HCl extract (corresponding to the arginine-rich and lysine-rich histones respectively of mammalian tissues[22]) was higher in the lower hypocotyl (8 cm from hook, ratio of ethanol–HCl extracted histone to HCl-extracted histone: 1·581; 4 cm from hook, ratio 1·140) than in the less differentiated (hook) tissue (ratio, 0·588). These results correspond with the earlier findings of Agrell and Christenson[6] that with development of embryonic cells, the ratio of the arginine-rich histones to lysine-rich histones increased.

Figure 1 shows the gel patterns of the total histones following 2·0 M NaCl extraction. These histones were neither dialyzed nor purified but were applied directly to the columns. Some bands are common to both stages of development of the hypocotyl while others appear to be tissue specific. Purification of these proteins by acetone precipitation and dialysis altered the gel patterns and reduced the differences between the two tissues (Fig. 2, cols. 5 and 6). It should be noted that the hook gel in column 6 differs in pattern from the hook gel in Fig. 1. The difference is probably due to two factors: (1) The precipitated and dialyzed histones in Fig. 2 were purified and concentrated more than those in Fig. 1. In fact, following precipitation of total histone, not all protein could be dissolved, suggesting that contaminat-

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ing proteins were removed by this step. (2) The high concentration of NaCl in the case of total histones in Fig. 1 could have altered the pattern. Columns 1 through 4 show patterns of the HCl-extracted and ethanol–HCl fractions. Histones extractable with ethanol–HCl (cols. 3 and 4) were found in small quantities in the plant tissue and gave inconsistent results upon electrophoresis, primarily because they failed to enter the gel columns unless current was applied for a long time and sample solutions were high in ionic strength (*ca.* 0·5 M). Such difficulties have been described previously.[23–24] Total histone (Fig. 2, cols. 5 and 6) showed some bands not present in the gels of the ethanol–HCl or HCl histones. Apparently these proteins were denatured during isolation of the two fractions of histones using alcohol–HCl solvents. Columns 5 and 6 suggest that there is almost no tissue specificity for a given stage of development; in addition it should be noted that the histones were extracted from crude chromatin which may be contaminated with other cellular components.

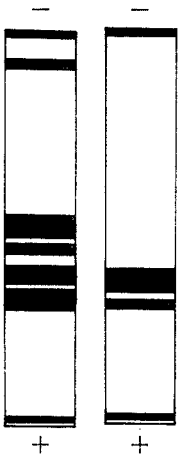

FIG. 3. ACIDIC CHROMOSOMAL PROTEINS FROM CHROMATIN OF HOOK (left) AND LOWER HYPOCOTYL (right). The gels are 15%, pH 8·9. Top: cathode; bottom: anode. 400 μg protein/column.

Acidic Nuclear Proteins. The acidic chromosomal proteins were found to have patterns specific to the stage of development (Fig. 3). The specificity of acidic nuclear proteins for developing tissues suggests that these proteins may play a role in the differentiation of cells. The close association of acidic nuclear proteins with nucleic acids in the chromatin of cells at interphase implicates them as possible components of gene regulating systems. It is of interest that pattern differences between the undifferentiated (hook) tissue and the more differentiated (lower hypocotyl) tissue were greater among acidic proteins than among the histones. Crude chromatin as the source of nuclear proteins certainly contained some cytoplasmic contaminations; the methods of extraction, however, were somewhat selective for the specified acidic or basic proteins. The precipitated histones were not soluble in dilute alkali and the precipitated acidic proteins were almost insoluble in dilute acidic solutions. The histones did not migrate toward the anode in the 15 per cent, pH 8·9, gel system, and the acidic proteins did not migrate toward the cathode in the 15 per cent, pH 2·9, gel system.

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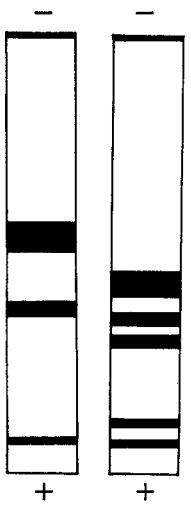

FIG. 4. ACIDIC RIBONUCLEOPROTEINS OF CHROMATIN OF HOOK (left) AND LOWER HYPOCOTYL (right). The gels are 15%, pH 8·9. Top: cathode; bottom: anode. 300 μg protein/column.

EXPERIMENTAL

Bush beans (*Phaseolus vulgaris*, variety Burpee's Stringless Greenpod) were grown in the dark in vermiculite at 23° for 7 days. 1 cm hypocotyl segments were excised from the uppermost meristematic portion (the "hook") and from a section 8 cm below the hook (the "lower" hypocotyl). The latter segment represents a relatively more differentiated tissue than the hook. The segments were rinsed in distilled water and floated in 0·5 M $CaCl_2$ for half an hour at 4° to stiffen the cell walls. The segments were then rinsed in distilled water and used immediately. An alternative method for facilitating cell-wall destruction was freezing the tissue at −20° before extraction.

Isolation of Chromatin

All isolation and purification procedures for each group of segments (hook and lower hypocotyl) were the same and, unless noted otherwise, were performed at 4°. Approximately 600 g of each group of segments was placed in a chilled Waring blendor with a sucrose-buffer solvent (1 part tissue–4 parts solvent, w/w). This solvent, called the TKMC buffer, consisted of 0·4 M sucrose, 0·025 M KCl, 0·005 M $MgCl_2$, and 0·002 M $CaCl_2$ in 0·05 M Tris-HCl, pH 7·5. The segments were ground for 2 min at 6000 rev/min after which the slurry was poured through four layers of cheese-cloth. The filtrate was centrifuged 10 min in a Sorvall RC-2 refrigerated centrifuge at $1000 \times g$ to sediment intact cells, nuclei, and cell debris. The supernatant was then centrifuged for 20 min at $5000 \times g$ to sediment the chromatin. The chromatin was scraped off the underlying firm layer and homogenized in the TKMC buffer and centrifuged as above. The pellet of *crude chromatin* was saved for further extraction.

Protein Extraction

Procedure A. The chromatin was suspended with a teflon homogenizer in 2 vol of 2·0 M NaCl, pH 7·0. The slurry was transferred to a Sorvall stainless-steel "omnimixer" and homogenized 30 sec at 6000 rev/min. The homogenate was transferred to a centrifuge tube and centrifuged 20 min at $25{,}000 \times g$. The supernatant was saved and the residue extracted again as above. The supernatants were combined and represented the *deoxyribonucleoproteins.*

The residue was resuspended in 5 vol of 0·05 M NaOH for 4 hr and then centrifuged 20 min at $20{,}000 \times g$. The supernatant was saved and the residue extracted two more times as before. The combined supernatants represented the *acidic ribonucleoproteins.*

The deoxyribonucleoproteins in the 2·0 M NaCl extract were acidified with 5·0 N HCl to make the solution 0·25 N with respect to HCl. The solution was allowed to stand for 6 hr and was then centrifuged 20 min at $10{,}000 \times g$. The supernatant was saved and the pellet extracted again for 6 hr with 4 vol of 0·25 N HCl. The solution was centrifuged as above, and the supernatants, representing the *total histones*, were combined. The residue was then suspended in 2 vol of 0·1 N NaOH for 4 hr. The solution was centrifuged 20 min at $20{,}000 \times g$; the supernatant was saved and the pellet extracted again by the same procedure. The combined supernatants represented the *acidic chromosomal proteins.*

Procedure B. Here, the crude chromatin was first suspended in 5 vol of 10 per cent potassium acetate in 96 per cent ethanol to remove lipids.[25]

The mixture was allowed to stand for 4 hr after which it was centrifuged 30 min at 10,000 × *g*. The supernatant was discarded and the residue suspended by the aid of a teflon homogenizer in 4 vol of ethanol–HCl mixture (80 per cent ethanol, 20 per cent 1·25 N HCl, v/v) and homogenized briefly. After 6 hr the suspension was centrifuged 20 min at 20,000 × *g*. The supernatant was saved and the residue again extracted as before. The supernatants were combined and represented the *ethanol*-HCl *histone fraction.* It is known that in extractions of animal tissue this fraction would contain the arginine-rich histones. The residue from this extraction was suspended and homogenized in 4 vol of a 0·25 N HCl solution. The mixture was allowed to stand for 6 hr and then centrifuged 20 min at 20,000 × *g*. The supernatant was saved, and the residue extracted again by the same procedure. The combined supernatants represented the HCl *histone fraction.* In animal tissue, this fraction would contain the lysine-rich histones.

Protein purification. The acidic ribonuclear proteins, and the acidic chromosomal proteins were purified by a modification of the method of Busch and Steele.[26] The proteins were precipitated from their alkaline extracts by carefully lowering the pH to 5·0 with dil. HCl. The resulting suspensions were centrifuged 20 min at 10,000 × *g*. The sedimented proteins were suspended in 0·05 N NaOH and allowed to stand for 1 hr. They were then centrifuged as above to remove any undissolved protein. The solutions of the acidic chromosomal fractions were precipitated and suspended only once, while the acidic ribonuclear protein fraction was precipitated two more times by the same procedure.

The total histone fraction from the 2·0 M NaCl extract as well as the ethanol–HCl and HCl fractions of the crude chromatin were partially purified by a modification of the methods of Johns and Butler.[27] The total histone fraction was first dialyzed against 0·01 N HCl for 12 hr at 4° with constant stirring and intermittent changes of the dialyzing medium. The dialyzed total histone and the ethanol–HCl and HCl fractions were now treated similarly, except that the pH of the ethanol–HCl fraction was raised to 10. To each fraction were added 5 vol of acetone (−20°); the suspensions were allowed to stand for 16 hr at −20°. The flocculent mixtures were centrifuged at 10,000 × *g* for 20 min at −10°. The supernatants were discarded and the pellets were dried in a vacuum at −20°. The dry sediments were then suspended in 0·25 N HCl for several hr and then centrifuged at 10,000 × *g* for 20 min at 4° to remove any undissolved protein.

Measure of protein quantity. Protein concentration was determined by the method of Warburg and Christian[28] and by the method of Lowry and co-workers.[21] The spectrophotometric method was used during isolation and purification. We have found that with purified preparations the two methods differ by about 5 per cent. However, it is extremely important that proper standards are used. Bovine serum albumin may be used as a standard for all acidic and nuclear sap proteins. Histone must be used as a standard for measurement of histone. Both methods were used to measure protein in the fractions after purification.

Electrophoresis of Nuclear Proteins

1. *The 15 per cent, pH 8·9 gel system.* This system was used to separate the acidic ribonuclear and acidic chromosomal proteins and was similar to that of Ornstein[29] except that the gel was made by mixing solutions A, C and G in the proportion of 1:4:3:

Solution A; 1·0 N HCl, 24·00 ml; Tris (2-amino-2-hydroxymethyl-1,3-propanediol, 18·15 g); Temed (N,N,N′-tetramethylethylene-diamine), 0·12 ml; and water up to 100·00 ml (pH 8·9).

Solution C; Acrylamide, 30·00 g; *Bis*, 0·40 g; and water up to 100·00 ml (pH 8·9).

Solution G; Ammonium persulfate, 0·14 g in water (100 ml), pH 8·9.

Proteins were electrophoresed in columns 3·5 × 0·5 cm at 3·0 ma per column. The proteins were stained with 0·5% buffalo blue black in 7% acetic acid.

2. *The 15 per cent, pH 2·9 system.* This system was used to separate the histone fractions. The separating gel is suited for proteins or peptide molecules of a mol. wt. of 2000–30,000. This system stacks proteins at pH 4·0 and separates them at pH 2·9. These conditions were better suited for basic proteins, especially histones which have a tendency to aggregate at higher pH. The buffer and gel solutions were as follows:

Solution A; 1·0 N KOH, 00 00 ml; Glacial acetic acid, 35·47 ml; Temed, 0·77 ml; with water up to 50·00 ml, pH 2·9. *Solution C;* As above but at pH 2·9. *Solution G;* $(NH_4)_2S_2O_5$, 1·40 g; in water (100.00 ml), pH 2·9. *Solution H;* Glycine, 28·10 g; Glacial acetic acid, 3·06 ml; with water; 1000·00 ml, pH 4·0.

The separating gel was made by mixing solutions A, C, and G in the proportions of 3:4:1 respectively and adjusting the pH to 2·9. The procedures for setting up the gel columns were the same as Ornstein,[29] and the gel was placed in larger glass tubes with an i.d. of 0·7 cm and a length of 6·0 cm. The gels were filled to a level of 5·0 cm. Migration of proteins was toward the cathode. No marker band was used. The current applied was 6 Ma/column for 3 hr. The gels were then removed and treated as the other gels.

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