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ISOLATION AND ANALYSIS OF THE PROTEINS OF PLANT NUCLEI FROM TWO STAGES OF DIFFERENTIATION OF *PHASEOLUS VULGARIS**

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Abstract—The proteins of plant nuclei from *Phaseolus vulgaris* were separated and analyzed by polyacrylamide gel electrophoresis and amino acid analysis. Comparative studies of some protein fractions revealed quantitative and qualitative differences between the undifferentiated hook and the more differentiated lower hypocotyl of bean. While the histone fractions displayed similar electrophoretic patterns, the acidic protein fractions varied accordingly to the stage of development. Various protein fractions from the same tissue differed markedly in amino acid composition; there was little variation in similar fractions from the two stages of differentiation.

INTRODUCTION

THE ROLE of nuclear proteins other than enzymes is largely unknown at present. The nucleohistones have received wide attention and their role as template suppressors is generally accepted; however, their prime role in gene regulation and in differentiation is not clear. Recent investigations have shown that histones exhibit neither a tissue specificity nor a specificity for the active or inactive areas of chromosomes.[1-3] These results cast some doubt on the possibility that histones are the primary gene regulators. The acidic proteins represent a large portion of the total nuclear proteins.[4] The association of these proteins with nucleic acids and their high rate of turnover has implicated them as potential gene regulators.[1]

The acidic nuclear proteins associated with the chromatin of bean hypocotyls were shown to exhibit some tissue specificity.[5,6] However, crude chromatin was the source of nuclear proteins and consequently contamination by cytoplasmic components could not be ruled out.

In this paper we described isolation of proteins from highly purified nuclei of bean tissues; the procedures[1,7-12] were modified for use with plant nuclei. Nuclear proteins from two

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[1] L. S. HNILICA, *Prog. Nucl. Acid Res.* **77**, 25 (1967).
[2] J. H. FRENSTER, V. G. ALLFREY and A. E. MIRSKY, *Proc. Natl. Acad. Sci. (U.S.A.)* **50**, 1025 (1963).
[3] J. H. FRENSTER, *Nature* **206**, 680 (1965).
[4] H. BUSCH, *Histones and Other Nuclear Proteins*, p. 198, Academic Press, New York (1965).
[5] I. V. SARKISSIAN and T. C. SPELSBERG, *Physiol. Plant.* **20**, 991 (1967).
[6] T. C. SPELSBERG and I. V. SARKISSIAN, *Phytochem.* **7**, 2083 (1968).
[7] V. G. ALLFREY, A. E. MIRSKY and H. STERN, *Adv. Enzymol.* **16**, 411 (1955).
[8] W. R. KIRKHAM and L. E. THOMAS, *J. Biol. Chem.* **200**, 53 (1953).
[9] A. E. MIRSKY and POLLISTER, *J. Gen. Physiol.* **30**, 117 (1946).
[10] A. O. POGO, B. G. T. POGO, V. C. LITTAU, V. G. ALLFREY, A. E. MIRSKY and M. G. HAMILTON, *Biochim. Biophys. Acta* **55**, 849 (1962).
[11] W. J. STEELE and H. BUSCH, *Cancer Res.* **23**, 1153 (1963).
[12] I. B. ZBARSKII and O. P. SAMORINA, *Biokhimiya* **27**, 556 (1962).

developmentally different sections of hypocotyl of beans are compared under conditions of minimum contamination by cytoplasmic materials. This study demonstrates the feasibility of extraction and analysis of nuclear proteins of plant tissue. Such knowledge would appear to be important as attempts are continually directed toward description of biochemistry of plant nuclei with the ultimate aim of learning the biochemical basis of differentiation.

RESULTS AND DISCUSSION

After the initial sedimentation from tissue extracts, the nuclei were associated with much cytoplasmic debris which appeared as fine threads which stained darkly with aceto-orcein. Sedimented through 2·2 M sucrose, the nuclei were almost completely free of contaminating debris. The nuclei of the hook section were stained more darkly with aceto-orcein than those of the lower hypocotyl. Nuclei of the latter tissue appeared to vary in size more than those of the hook tissue and were generally larger.

Protein Composition of Nuclei

Table 1 shows the total quantity of each protein fraction as the per cent of the total protein extracted from the nuclei of the two tissues. The more differentiated lower hypocotyl

TABLE 1. PROTEINS OF NUCLEI FROM BEAN TISSUES

Protein fraction	Lower hypocotyl	Hook
Nuclear sap	14–18*	20–28*
Acidic ribosomal	10–18	16–25
Histones	2–9	3–7
Acidic chromosomal	3–9	3–6
Acidic ribonuclear	53–58	30–38

* Figures represent per cent of total protein.

had a much greater amount of acidic ribonuclear proteins than the less differential hook; however, the lower hypocotyl had less nuclear sap and acidic ribosomal proteins. Equal amounts of histones and acidic chromosomal proteins were found in both stages.

Nuclear Sap Proteins

The nuclear sap proteins proved to be very heterogeneous. Figure 1 shows that some nuclear sap protein bands are specific for the particular stage of development while others are common to both stages.

The more complex protein pattern of the nuclei of lower hypocotyl is not surprising since the cells of this more differentiated area exhibit a greater variation in structure and function than do those of the hook. The proteins of the nuclear sap of the two stages of development followed a similar pattern as the cytoplasmic proteins.[5, 13]

Acidic Ribosomal Proteins

The acidic ribosomal proteins also exhibited some heterogeneity although not as much as the nuclear sap proteins (Fig. 1). The patterns of the two stages of development are different; some protein bands are common to both tissues while others exhibit tissue specificity.

[13] I. V. SARKISSIAN and T. C. SPELSBERG, *Nature* **214**, 319 (1967).

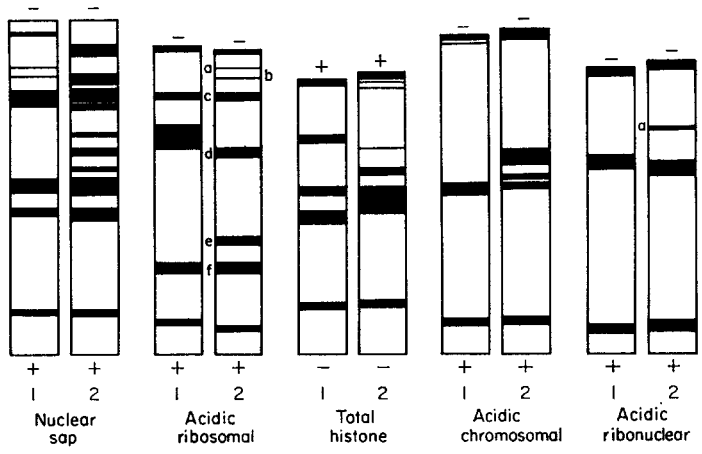

FIG. 1. POLYACRYLAMIDE GEL PATTERNS OF THE NUCLEAR PROTEINS FROM PURIFIED NUCLEI. For each fraction column 1 represents proteins from the undifferentiated tissue (hook) and column 2 represents the proteins from the more differentiated lower hypocotyl. Direction of migration is from top to bottom; the polarity of the electrodes is indicated by signs. 300 μg protein per column.

Band "e" in the hypocotyl was always found in the patterns of the acidic ribonuclear proteins when extraction with Tris buffer was omitted. Bands "a"–"d" could be detected only when the Tris extraction was included. Band "d", while present in both tissues, was found in much higher proportion in the nuclei of the hook. Table 2 gives the amino acid composition of the acidic ribosomal proteins of nuclei of both tissues. The proteins appear to be very acidic but do not appear to differ in their amino acid composition.

Histones

The gel columns of the total histones, extracted from the deoxynucleoproteins of isolated nuclei of both tissues, and purified by acetone precipitation, are shown in Fig. 1. The patterns are strikingly similar, but some quantitative differences in bands in the two stages of development are observed. This observation would be caused by proteolytic action during the isolation.[14, 15] Several faint bands were found to be tissue specific while others appeared to be common to both stages of development. These proteins, however, may not be all histones, which is supported by the amino acid composition (Table 2).

The overall nature of the histone fractions of both tissues appears to be acidic. It appears, therefore, that these histone fractions are contaminated with acidic proteins. This is not surprising since the histones isolated for amino acid composition were extracted from crude chromatin instead of the purified nuclei. Chromatin was used because the yield of crude chromatin is much greater from the tissues than from purified nuclei. Consequently, histones could be extracted in sufficient amounts for each of the subfractions to be analyzed for amino acid composition.

Acidic Chromosomal Proteins

The acidic chromosomal proteins were found to have patterns specific to each stage of development. Electrophoretic patterns of these proteins are shown in Fig. 1. The amino acid composition of the acidic chromosomal proteins of both tissues is shown in Table 2.

[14] S. PANYIM, R. H. JENSEN and R. CHALKLEY, *Biochim. Biophys. Acta* **160**, 252 (1968).
[15] R. H. STELLWAGEN, B. R. REID and R. D. COLE, *Biochim. Biophys. Acta* **155**, 581 (1968).

TABLE 2. AMINO ACID COMPOSITION OF PROTEIN FRACTIONS OF HOOK AND LOWER HYPOCOTYL OF BEAN*

	Acidic			Histones	
Amino acids	Ribosomal	Chromosomal	Ribonuclear	Ethanol–HCl	HCl
HOOK					
Hydroxyproline	—	—	—	—	1
Aspartic acid	11	10	18	16	8
Threonine	6	5	8	3	5
Serine	6	6	—	7	6
Proline	7	8	5	—	4
Glutamic acid	13	11	12	7	9
Glycine	10	9	9	8	13
Alanine	9	9	9	6	14
Valine	8	7	8	3	6
Methionine	1	1	<1	2	4
Isoleucine	5	5	5	4	5
Leucine	10	10	11	5	7
Tyrosine	3	3	4	3	3
Phenylalanine	4	4	4	4	3
Hydroxylysine	—	—	—	9	<1
Lysine	2	3	1	17	10
Arginine	5	4	4	5	3
% Recovery from hydrolysis	95%	88%	94%	82%	90%
LOWER HYPOCOTYL					
Hydroxyproline	—	—	—	—	1
Aspartic acid	12	9	18	17	9
Threonine	7	6	8	2	6
Serine	6	6	—	8	6
Proline	6	6	5	—	7
Glutamic acid	12	10	9	7	11
Glycine	10	10	9	7	8
Alanine	10	9	10	6	10
Valine	7	6	8	3	7
Methionine	1	1	<1	12	2
Isoleucine	5	5	6	5	5
Leucine	10	9	11	5	5
Tyrosine	3	4	4	4	3
Phenylalanine	4	5	4	5	4
Hydroxylysine	—	—	—	4	<1
Lysine	2	7	1	7	9
Arginine	5	5	4	6	4
% Recovery from hydrolysis	94%	85%	91%	85%	93%

* Per cent of total amino acids recovered.

The proteins of these fractions appear to be acidic in nature but not as much as the acidic ribosomal proteins. Only slight differences in amino acid composition were found between the proteins of the two stages of development.

Acidic Ribonuclear Proteins

The acidic ribonuclear proteins also show specificity to the stage of development. Figure 1 presents the gel columns of the purified proteins of both stages. The proteins show electrophoretic patterns which are specific to the stage of development, in that band "a" is found only in the proteins from the nuclei of the lower hypocotyl. Amino acid analysis of these

proteins is shown in Table 2. The proteins appear to be the most acidic of all the protein fractions. The proteins of the two stages of development do not appear to differ in amino acid composition.

Nuclear Proteins and Development

It has been shown by electrophoresis that two areas of hypocotyl of bean seedlings—the hook and the lower hypocotyl—have some nuclear proteins in common, and some which are specific to a particular segment of the hypocotyl. The histones appeared strikingly similar in the two stages of development. The amino acid compositions of identical protein fractions from the two stages did not differ markedly. It should be noted that a difference in electrophoretic patterns of two fractions of protein does not necessarily stem from differences in the total amino acid composition of the fractions.

The specificity of certain nuclear proteins for particular stages of development suggests that these proteins are somehow involved in the process of differentiation. Whether these proteins are involved in an active role, as enzymes or as repressors, or in a more passive role, as structural proteins in cell nuclei, remains to be learned. The specificity of the acidic proteins, but not the histones, for different stages of development suggests that the acidic proteins have a functional role in cell differentiation whereas the histones probably play a more passive role in regulation of nuclear activities. In any case, the tissue specificity of the nuclear proteins does indicate a tissue specific restriction of the DNA (i.e. tissue specific functional genes). The procedures described here for the extraction of the nuclear proteins may be inadequate; many protein species, especially the non-histone proteins associated with DNA, may not have been extracted or may have been denatured by the extraction procedures. Further investigation into this problem of isolation is needed. The patterns of chromosomal proteins with purified nuclei as source expectedly show considerably less complexity than those of the chromosomal proteins obtained earlier[6] from crude chromatin.

EXPERIMENTAL

Bush beans (*Phaseolus vulgaris*, cv. Burpee's Stringless Greenpod) were the source of nuclei. The growing of the plants and excision of relatively undifferentiated hook and differentiated lower hypocotyl has been described.[6] In the present experiments, the excised tissues were not frozen, but were used immediately.

Isolation and Purification of Nuclei from Segments of Bean Hypocotyls

All isolation and purification procedures[6] for each group of segments were the same, and were performed at 4°. Each group of segments (600 g) was placed in a Waring Blendor with standard TKMC buffer, 0·05 M Tris-HCl, pH 7·5; 0·025 M KCl; 0·005 M $MgCl_2$; 0·002 M $CaCl_2$; and 0·4 M sucrose, (1 part tissue: 4 parts buffer). The segments were ground for 1 to 2 min at 6000 r.p.m. The slurry was poured through four layers of cheese-cloth. The filtrate was saved while the residue was again blended in the TKMC buffer and filtered. The residue was discarded, and the combined filtrates were centrifuged 10 min at 200 *g*. The pellet was discarded and the supernatant containing intact nuclei and chromatin was centrifuged 20 min at 1000 *g*. The supernatant was saved and the pellet of nuclei was immediately suspended in the TKMC buffer. Unless stated otherwise, all suspensions were carried out with a power-driven teflon homogenizer. The nuclear suspension was then centrifuged 30 min at 600 *g*. The pelleted nuclei were suspended in the TKMC buffer and subjected to further purification.

The original supernatant from the first nuclear sedimentation was centrifuged 20 min at 5000 *g* to sediment the chromatin released from nuclei which had been ruptured by the isolation procedure. The chromatin was suspended in the TKMC buffer, centrifuged again at 500 *g* and the pellet was saved for further fractionation.

The suspension of nuclei was made in 1·6 M sucrose by addition of the appropriate amount of 2·2 M sucrose in TKMC buffer. The suspension was then placed in a layer over 2·2 M sucrose in TKMC buffer and centrifuged 20 min at 140,000 *g* in IEC rotor SM-498 in the B-60 ultracentrifuge. The sedimented nuclei were suspended in the standard TKMC buffer and were then ready for extraction. The nuclei were checked microscopically and found to be essentially free of cytoplasmic debris.

Extraction of Protein

The pelleted nuclei were suspended in 5 volumes of 0·14 M NaCl and 0·01 M Na-citrate, pH 7·0, and homogenized at high speed for 2 to 3 min. The mixture was stirred for 30 min and then was centrifuged 20 min

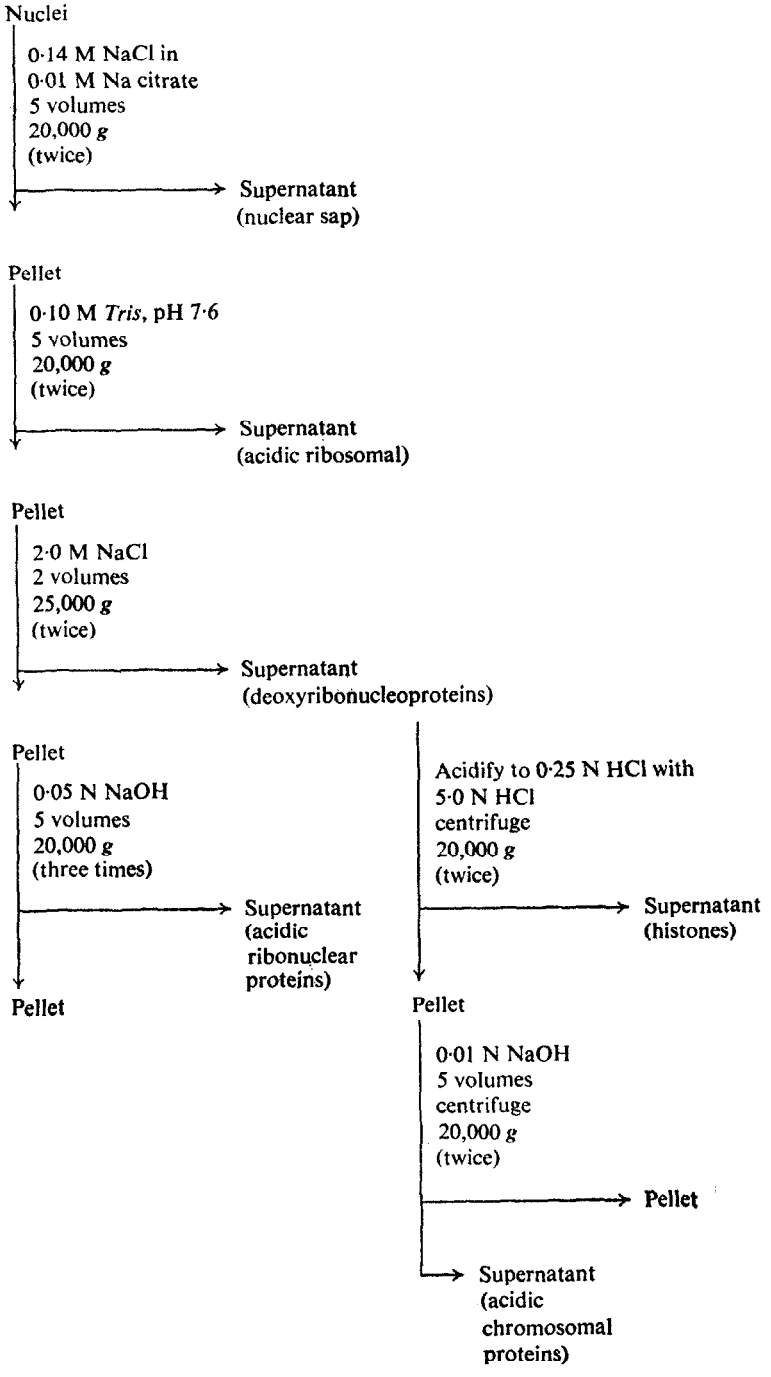

FIG. 2. PROCEDURE FOR EXTRACTION OF PROTEINS FROM THE NUCLEI OF BEAN HYPOCOTYL.

at 20,000 *g*. The supernatant was saved and the residue was again extracted and sedimented as above. The combined supernatants represented the *nuclear sap proteins.*

The residue from above was homogenized briefly with 5 volumes of 0·1 M Tris-HCl buffer, pH 7·6. After mixing for 1 hr, the homogenate was centrifuged 20 min at 20,000 *g*. The supernatant was saved and the pellet suspended, extracted, and centrifuged as above. The combined supernatants represented the *acidic ribosomal proteins.*

The extraction of acidic ribonuclear, histones, and the acidic chromosomal proteins has been described.[6] Procedures for extraction of protein from purified nuclei is shown in Fig. 2. The ethanol–HCl and HCl histones, corresponding to the arginine-rich and lysine-rich histones respectively in mammals, were extracted[6] from the crude chromatin which was isolated from the original homogenate of the tissues. The yield of nuclei from the tissues was sufficiently low that the two subfractions of histones were not isolated in amounts sufficient for analysis of heterogeneity and amino acid composition.

Purification of Nuclear Proteins

The nuclear sap and ribosomal proteins were precipitated by adding 3 volumes of 100% acetone at −20°. The suspensions were allowed to stand for 1 hr, after which they were centrifuged 10 min at 20,000 *g* at −10°. The supernatants were discarded and the pellets lyophilized. The powders were then suspended in their respective solvents (0·14 M NaCl, pH 7·0, in case of the nuclear sap fraction and 0·1 M Tris-HCl buffer, pH 7·6, in case of the ribosomal fraction). The suspensions were allowed to stand for 1 hr, after which they were centrifuged 10 min at 20,000 *g* at 4° to remove any denatured (undissolved) protein.

The acidic proteins, i.e. the acidic ribonuclear proteins and the acidic chromosomal proteins, were purified as described.[6] Protein concentration was determined by the method of Warbrug and Christian[16] and by the method of Lowry and co-workers.[17]

Amino Acid Analysis

Purified protein pellets from the hook and lower hypocotyl were suspended in 2 volumes of acetone. The mixtures were dried in a vacuum aspirator and the process repeated. Two 10 ± 0·1 mg samples of each of the dried protein samples were used, one for micro-Kjeldahl determination, and the other for amino acid analysis.

The sample for amino acid analysis was placed in a Thunberg tube; 1 ml conc. HCl and 1 ml deionized water were added to each tube. Each tube was brought to −20° and the contents then placed under high vacuum. The mixtures were hydrolyzed in an oven for 24 hr at 110 ± 2°. The analysis was performed on a Beckman 120-C amino acid analyzer.

Polyacrylamide Gel Disc Electrophoresis

The nuclear sap and acidic ribosomal proteins were electrophoresced in standard gel columns of 7·5% acrylamide, pH 8·9.[18] The acidic chromosomal and acidic ribonuclear proteins as well as histones were separated on special 15% gels.[6]

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[16] O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1942).
[17] O. H. LOWRY, J. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[18] B. J. DAVIS, *Ann. Acad. Sci. N.Y.* **121**, 404 (1964).