Plant Physiol. (1983) 71, 777–779
0032-0889/83/71/0777/03/$00.50/0

Pigment Analysis of Chloroplast Pigment-Protein Complexes in Wheat

Received for publication September 24, 1982 and in revised form December 2, 1982

KENNETH ESKINS, MURRAY E. DUYSEN, AND LINDA OLSON
Northern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois 61604 (K. E.); and Department of Botany, North Dakota State University, Fargo, North Dakota 58102 (M. E. D., L. O.)

ABSTRACT

Pigment-protein complexes separated from wheat (*Triticum aestivum* L. selection ND96-25 by two gel electrophoresis techniques were analyzed by high-performance liquid chromatography for chlorophylls and carotenoids. The two techniques are compared, and pigment analyses are given for the major reaction centers and light-harvesting complexes. Reaction centers contain mostly chlorophyll *a*, carotene, and lutein, whereas light-harvesting complexes contain chlorophyll *a*, chlorophyll *b*, lutein, and neoxanthin. The amounts of violaxanthin are variable.

The early work (10, 13) that established the presence of two major types of thylakoid pigment-protein complexes (CPI and CPII)[1] has been expanded considerably by more recent investigations (1, 2, 9, 14). Improved resolution has increased to at least seven the number of electrophoretic bands associated with specific photosystems (1, 6). Simultaneously, results of a search for techniques that produce smaller amounts of free pigment suggest that there may be fewer than seven distinctly different complexes (8, 9). Whereas some bands may be combinations of two or more bands seen by high resolution methods, it remains to be seen whether fewer and larger complexes accurately represent the natural state of pigment in the chloroplast.

Techniques used by Anderson and others generally produce seven bands as follows: (a) CPIa-associated with PSI, possibly a dimer of CPI or an association of CPI with light-harvesting complex (14); (b) CPI-associated with PSI reaction center, contains no Chl *b*; (c) LHPP1, LHPP2, LHPP3-oligomers, and monomer of light-harvesting pigment-protein complex, Chl *a*/*b* of 1.1 to 1.9; (d) CPa-associated with PSII reaction center, measured Chl *a*/*b* of 2.5 to 7.0 but probably contains no Chl *b* in pure preparations (14); (e) FP-variable content of free pigment depending on conditions used, Chl *a*/*b* of 2.7 to 4.4 (14) or no Chl *b* (1).

Electrophoretic bands produced by Markwell's techniques are less well-characterized and are called A-1, AB-1, AB-2, AB-3, and FP (8) or N, O, P, Q, and F (9). A-1 or N reportedly corresponds to CPI of Anderson (1). AB-1, AB-2, and AB-3 all contain Chl *b* and have mol wt of 80,000, 60,000, and 46,000 D, respectively. These bands do not correspond to O, P, and Q. In this latter system, P is the predominate Chl *b* complex and O and Q contain mostly Chl *a*. In terms of mol wt, AB-3 corresponds to Q and AB-2 to P, but both O and N are larger than A-1.

[1] Abbreviations: CP, Chl-protein(s); LHPP, light-harvesting pigment-protein(s); FP, free pigment.

Our approach was to compare detailed analyses of pigment-protein complexes prepared by methods of both Anderson and Markwell (1, 9). We wanted to pay particular attention to those chloroplast carotenoids, neoxanthin, violaxanthin, lutein, and carotene, which until recently have been largely ignored in discussions of pigment-protein complexes. Several researchers have acknowledged the presence of carotenoids while reporting analytical data for only Chl *a* and Chl *b*. This neglect of carotenoids was partially corrected by Rawyler *et al.* (11), who used TLC to make preliminary assignments of carotene to the Chl *a* complexes (CPI and CPa) and of the xanthophylls to the light-harvesting complexes. Their preliminary work has been further confirmed and expanded by Lichtenthaler *et al.* (7) using HPLC to analyze the pigments of radish chloroplast. Concurrently, we have associated certain carotenoids with specific pigment-protein complexes (4, 5), also using HPLC techniques. Herein, we report our analysis of

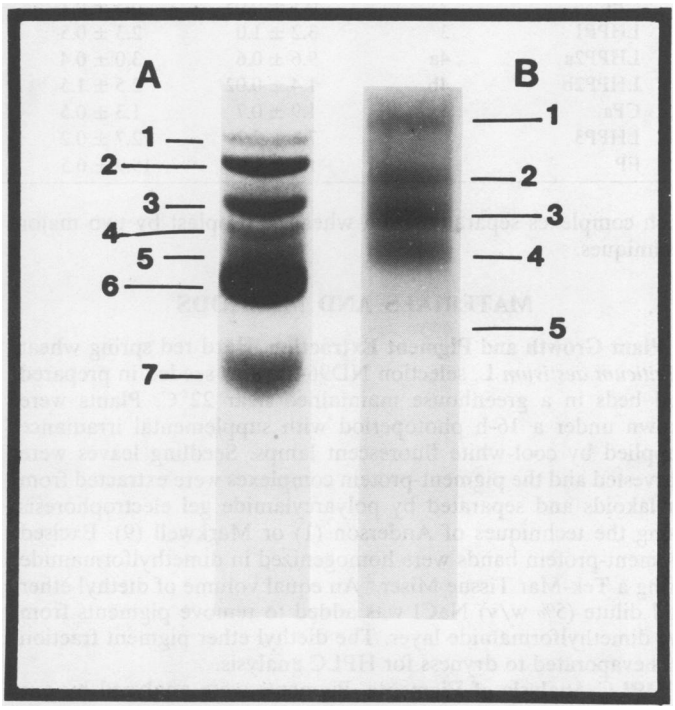

FIG. 1. Pigment protein complexes separated by polyacrylamide gel electrophoresis. A, Electrophoretogram according to Anderson's technique (9). Bands are: 1, CP1a; 2, CPa; 3, LHPP1; 4, LHPP2; 5, CPa; 6, LHPP3; 7, FP. B, Electrophoretogram by Markwell's method (9). Bands are: 1, A-1; 2, AB-1; 3, AB-2; 4, AB-3; 5, FP.

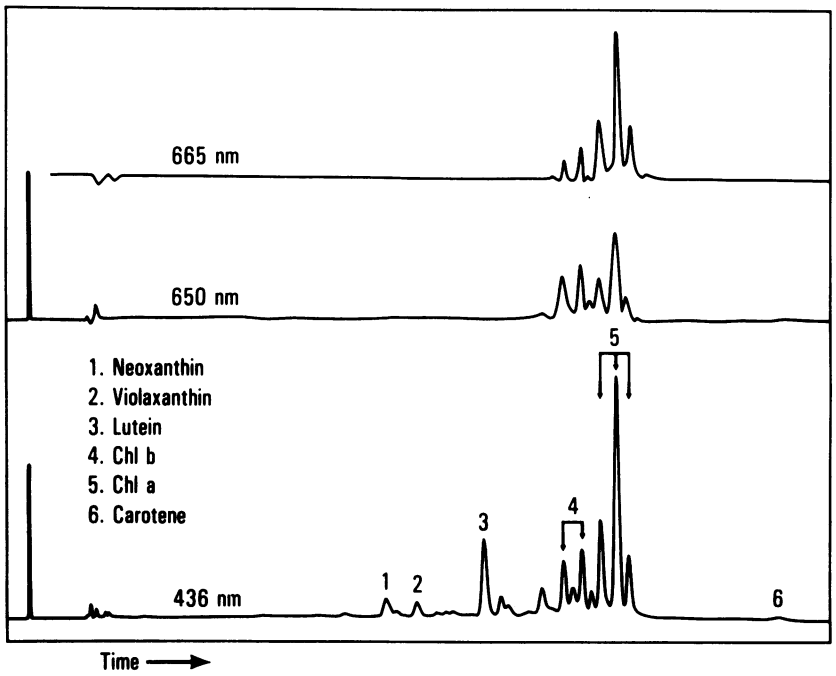

FIG. 2. HPLC scan of pigments neoxanthin, violaxanthin, lutein, Chl *b*, Chl *a*, and carotene at wavelengths 436, 650, and 665 nm. Pigments are derived from an extraction of light-harvesting pigment-protein three (LHPP3) separated by the method of Anderson (1).

Table I. *Ratio of Accessory Pigments to Chl a in Pigment-Protein Complexes of Wheat—Anderson's Method (1)*

Pigment Protein Complex	Band No.	Pigment Content					
		Neox	Violax	Lutein	Carotene	Chl *b*	Chl *a/b*
		nmol/100 nmol Chl a					
CPIa	1	0.2 ± 0.2	1.8 ± 0.9	2.2 ± 1.2	2.4 ± 0.8	20.7 ± 6.1	5.2 ± 1.5
CPI	2	0.1 ± 0.05	0.3 ± 0.1	1.0 ± 0.5	3.2 ± 1.7	7.5 ± 1.5	13.8 ± 2.8
LHPP1	3	3.2 ± 1.0	2.3 ± 0.5	10.1 ± 1.6	1.3 ± 1.1	64.4 ± 16.0	1.62 ± 0.4
LHPP2a	4a	9.6 ± 0.6	3.0 ± 0.4	25.1 ± 3.3	0.2 ± 0.4	86.3 ± 3.8	1.15 ± 0.05
LHPP2b	4b	1.4 ± 0.02	2.5 ± 1.5	5.9 ± 2.5	2.8 ± 1.3	48.1 ± 4.8	2.09 ± 0.2
CPa	5	1.9 ± 0.7	1.3 ± 0.5	5.2 ± 2.0	4.4 ± 1.3	33.8 ± 13.2	3.4 ± 1.5
LHPP3	6	7.3 ± 2.2	2.7 ± 0.2	17.2 ± 6.6	0.5 ± 0.4	83.3 ± 16.8	1.2 ± 0.2
FP	7	1.8 ± 1.0	15.9 ± 6.5	14.2 ± 4.9	5.3 ± 3.2	43.5 ± 10.0	2.4 ± 0.8

such complexes separated from wheat chloroplast by two major techniques.

MATERIALS AND METHODS

Plant Growth and Pigment Extraction. Hard red spring wheat (*Triticum aestivum* L. selection ND96-25) was seeded in prepared soil beds in a greenhouse maintained near 22°C. Plants were grown under a 16-h photoperiod with supplemental irradiance supplied by cool-white fluorescent lamps. Seedling leaves were harvested and the pigment-protein complexes were extracted from thylakoids and separated by polyarcylamide gel electrophoresis using the techniques of Anderson (1) or Markwell (9). Excised pigment-protein bands were homogenized in dimethylformamide using a Tek-Mar Tissue Miser.[2] An equal volume of diethyl ether and dilute (5% w/v) NaCl was added to remove pigments from the dimethylformamide layer. The diethyl ether pigment fraction was evaporated to dryness for HPLC analysis.

HPLC Analysis of Pigments. Pigments were analyzed by our previously published HPLC method (3). However, the analysis pattern showed multiple peaks in the area of Chl *a* and Chl *b*. It was necessary to scan at several wavelengths to establish the nature of these decomposition peaks. Scanning at 436 nm gave peaks for both carotenoids and Chl whereas scans at 650 and 665 nm indicated which peaks were derived from Chl *b* and Chl *a*, respectively. The results of individual analyses were recalculated using Chl *a* as an internal standard and were reported as nmol of accessory pigment per 100 nmol of Chl *a*. Usually, the results of four to six separate analyses were combined to give the reported value.

RESULTS AND DISCUSSION

Typical gel patterns for the two different electrophoretic methods are shown in Figure 1 (1a, Anderson's method; 1b, Markwell's method). Generally, Anderson's method gave seven peaks having the following percentages of total Chl: CPIa (10%), CPI (18%), LHPP1 (6%), LHPP2 (8%), LHPP3 (26%), CPa (10%), and FP (20%). Markwell's method gave five peaks: A-1 (17%), AB-1 (28%), AB-2 (26%), AB-3 (26%), and FP (5%). We have used the terminology proposed by both authors with the exception of referring to the complexes as pigment-proteins (PP) instead of Chl-proteins (CP) and to free pigment (FP) instead of free Chl (FC). This is meant to convey the presence of carotenoids as

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the United States Department of Agriculture over other firms or similar products not mentioned.

Table II. *Ratio of Accessory Pigments to Chl a in Pigment-Protein Complexes of Wheat—Markwell's Method (9)*

Pigment Protein Complex	Band No.	Pigment Content					
		Neox	Violax	Lutein	Carotene	Chl *b*	Chl *a/b*
		nmol/100 nmol Chl a					
A-1	1	0.06 ± 0.1	2.0 ± 0.2	2.5 ± 1.4	4.5 ± 2.6	16.2 ± 0.7	6.2 ± 0.3
AB-1	2	1.5 ± 0.4	1.9 ± 0.5	4.9 ± 0.5	5.5 ± 1.7	24.4 ± 6.1	4.2 ± 0.8
AB-2a	3a	7.4 ± 1.5	4.5 ± 0.9	14.4 ± 6.0	0.3 ± 0.2	77.7 ± 14.1	1.3 ± 0.2
AB-2b	3b	3.7 ± 1.2	2.5 ± 0.5	12.4 ± 3.0	3.2 ± 0.3	53.8 ± 11.6	1.9 ± 0.4
AB-3	4	4.8 ± 0.9	4.0 ± 0.8	8.6 ± 1.9	1.5 ± 0.5	74.9 ± 25.1	1.4 ± 0.4
FP	5	0.5 ± 0.6	8.4 ± 2.7	6.6 ± 2.2	2.9 ± 0.8	42.5 ± 18.4	2.6 ± 0.9

integral components of these complexes.

A typical chromatogram of pigments separated by HPLC is shown in Figure 2. Separation of carotenoids and various forms of Chl *a* and Chl *b* was observed. The various forms of Chl *a* and Chl *b* are similqr to those reported (12) for blanched and frozen spinach. It is not completely clear whether the decomposition of the Chl was a direct result of the electrophoretic technique, but samples of pigment treated the same in all ways except for electrophoresis show minor amounts of decomposition. Both Anderson's and Markwell's procedures resulted in multiple Chl peaks. Various attempts to prevent decomposition by including antioxidants and free radical traps in the gel and extraction media were unsuccessful. In our analysis, we have assumed that the various forms of both Chl *a* and Chl *b* are equivalent to their parent forms. The combined analysis of at least four different runs is shown for Anderson's technique in Table I and for Markwell's technique in Table II. Also shown are the average ratios of Chl *a* to *b* for each of these bands.

First, we may conclude from these results that both methods give bands containing significant quantities of carotenoids. Thus, carotene is a principal constituent of the reaction center complexes but is probably missing from the light-harvesting complexes. Neoxanthin, violaxanthin, and lutein are the principal carotenoids of the light-harvesting complexes, with a neoxanthin to violaxanthin ratio of 1.4 to 3.2. Lutein is a constituent of all complexes. Second, Markwell's method gives bands significantly different from those of Anderson, both in mol wt and in pigment composition. There is a correspondence between the pigment composition of several bands in the two methods, *i.e.* Markwell's Band A-1 appears to be a mixture of Anderson's CPIa and CPI, and AB-1 could be a complex of CPI and LHPP1, perhaps the PSI reaction center with its connecting light harvesting particle. AB-2a is similar to LHPP2a or a combination of LHPP1 and LHPP2a. AB-2b could be a complex of CPa with LHPP2 and AB-3 a complex of CPa and LHPP3. Obviously, further characterization of the proteins as well as pigments are needed to clarify these assignments. One may not yet conclude from this data, which method is superior or more accurately represents reality.

LITERATURE CITED

1. ANDERSON JM, JC WALDRON, SW THORNE 1978 Chlorophyll-protein complexes of spinach and barley thylakoids. FEBS Lett 92: 227–233
2. DUYSEN ME, LL OLSON, TP FREEMAN, ND WILLIAMS 1982 Photosynthetic properties of a wheat mutant deficient in pigment protein complexes. Photosynth Res In press
3. ESKINS K, HJ DUTTON 1979 Sample preparation for high-performance liquid chromatography of plant pigments. Anal Chem 51: 1885–1886
4. ESKINS K, L HARRIS, RL BERNARD 1981 Genetic control of chloroplast pigment development in soybeans as a function of leaf and plant maturity. Plant Physiol 67: 759–762
5. ESKINS K, WF KWOLEK, L HARRIS 1982 The accumulation of accessory pigments as a function of chlorophyll a. A comparison of development and genetic control. Physiol Plant 54: 409–413
6. HAYDEN DB, WG HOPKINS 1977 A second distinct chlorophyll a-protein complex in maize mesophyll chloroplast. Can J Bot 55: 2522–2529
7. LICHTENTHALER HK, U PRENZEL, G KUHN 1982 Carotenoid composition of chlorophyll-carotenoid-proteins from radish chloroplast. Z Naturforsch 37: 10–12
8. MARKWELL JP, S REINMAN, JP THORNBER 1978 Chlorophyll-protein complexes from higher plants. A procedure for improved stability and fractionation. Arch Biochem Biophys 190: 136–141
9. MARKWELL JP, JP THORNBER, RT BOGGS 1979 Higher plant chloroplast: evidence that all the chlorophyll exist as chlorophyll-protein complexes. Proc Natl Acad Sci USA 76: 1233–1235
10. OGAWA T, F OBATA, K SHIBATA 1966 Two pigment proteins in spinach chloroplast. Biochim Biophys Acta 112: 223–234
11. RAWYLER A, LEA HENRY, PA SIEGENTHALER 1980 Acyl and pigment lipid composition of two chlorophyll-proteins. Carlsberg Res Commun 45: 443–451
12. SCHWARTZ SJ, SL WOO, JH VON ELBE 1981 High-performance liquid chromatography of chlorophylls and their derivatives in fresh and processed spinach. J Agric Food Chem 29: 533–535
13. THORNBER JP, RPF GREGORY, CA SMITH, JL BAILEY 1967 Studies on the nature of the chloroplast lamella. I. Preparation and some properties of two chlorophyll-protein complexes. Biochemistry 6: 391–396
14. WILD A, B URSCHEL 1980 Chlorophyll-protein complexes of *Chlorella fusca*. Z Naturforsch 35: 627–637