ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 9, pp. 1021-1026. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © S. M. Kochubey, O. Yu. Bondarenko, V. V. Shevchenko, 2007, published in Biokhimiya, 2007, Vol. 72, No. 9, pp. 1255-1261.

A New Type of Subchloroplast Fragments Isolated from Pea Chloroplasts in the Presence of Digitonin

S. M. Kochubey*, O. Yu. Bondarenko, and V. V. Shevchenko

Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Vasilkovskaya str. 31/17, 03022 Kyiv, Ukraine; fax: (380) 44258-8146; E-mail: smk_off@naverex.kiev.ua

Received May 8, 2007
Revision received June 6, 2007

Abstract—Heavy fragments were isolated from pea chloroplasts using digitonin treatment and differential centrifugation. The particles were characterized by a significantly lowered chlorophyll *a/b* ratio, contents of photosystem I (PS I) proteins and ATPase, as well as of amount of P700. The content of photosystem II (PS II) proteins decreased insignificantly, whereas that of proteins of the light-harvesting complex II did not change. The absorption and low-temperature fluorescence spectra were indicative of a decreased content of PS I. Electron microscopy of ultrathin sections of heavy fragment preparations identified them as grana with reduced content of thylakoids. The diameter of these particles was practically the same as within chloroplasts. Comparison of various characteristics of the fragments and chloroplasts from which the fragments were isolated allowed us to define a high degree of preservation of marginal regions in thylakoids present in the heavy fragment particles. Analysis of the results shows that the procedure of fragmentation produces grana with high extent of thylakoid integrity. The phenomenon of reduction of the thylakoid content in grana, occurring as our heavy fragments, is considered in the frame of our previous hypothesis concerning the peculiarities of grana organization in the transversal direction.

DOI: 10.1134/S0006297907090155

Key words: chloroplast, grana, thylakoid, subchloroplast fragments

The dynamic properties of chloroplast ultrastructure are within the area of interest for modern investigations. In particular, a significant number of works in the 1980s and 1990s were devoted to the problems of lateral migration of the light-harvesting complex II (LHC II) of photosystem II (PS II) and partial destacking of granal thylakoids. Recently, ultrastructural changes called gross-changes have been described. For example, they include the phenomenon of a decrease of grana size or, in contrast, their aggregation happening during a short-term period [1, 2]. To reveal the mechanism of such events, it is necessary to have information concerning peculiarities of grana organization able to provide for such dynamics. However, to the present time grana are considered as a sufficiently conserved system, at least from the point of view of its arrangement in the direction perpendicular to the plane of thylakoids, i.e., in the transversal direction. Such idea is based on electron-microscopic investigations of 1970s and 1980s. More detailed information about peculiarities of grana organization could be obtained after their isolation from chloroplasts. However, there are practically no such works. Authors from [3] use chloroplast fragments obtained by sonication and called by them grana. This is the so-called B3 fraction. However, the described characteristics of this fraction [4] allow only to indicate that particles present in this fraction are the granal thylakoids which preserved of marginal regions with a certain extent of integrity. So, there are insufficient data to identify them with grana that retained its integrity including the transversal direction as well. However, just such fragments of chloroplast ultrastructure are of interest for the development of approaches for revealing the mechanisms of the gross-changes type dynamics.

Recently we have used solubilization by digitonin for isolation of heavy fragments of pea chloroplasts [5]. Testing some of their characteristics suggests that these particles are either chloroplasts with removed intergranal thylakoids or separate grana. This work deals with the detailed study of above-mentioned chloroplast fragments to reveal their nature and structural peculiarities.

Abbreviations: Chl) chlorophyll; LHC II) light-harvesting complex II; PS I, PS II) photosystems I and II.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Pea plants were grown in a vegetation area from May to September. Chloroplasts were isolated from leaves of the second layer of two-week-old pea plants. The leaves were homogenized for 2 min in 50 mM Tricine buffer, pH 7.6, 0.4 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, and then the homogenate was filtered and centrifuged for 5 min at 400*g*. The supernatant was centrifuged for 10 min at 1000*g*. The pellet was resuspended in 10 mM Tricine buffer, pH 7.6, 0.1 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl.

Heavy fragments were obtained by chloroplast solubilization with 0.3% digitonin for 15 min in the dark in an ice bath at the digitonin/chlorophyll ratio 3 : 1. The solubilized preparation was centrifuged for 10 min at 1000*g*. The pellet was resuspended in the medium that was used for chloroplasts. The yield of heavy fragments was from 17 to 30%. It was estimated as the ratio of total chlorophyll in the pellet to total chlorophyll in chloroplasts taken for fragmentation.

In the course of sample preparation for electron-microscopic measurements the chloroplast and fragment pellets were placed in agarose blocks, fixed with 3% glutaraldehyde solution in cacodylate buffer, followed by additional fixation with 1% osmium tetroxide solution in the same buffer. Then agarose blocks were embedded in Epon–Araldite mixtures and polymerized. Ultrathin sections of fixed material were obtained with a UMTP-6M microtome of Selmi (Russia) and stained with lead citrate. Digital microphotographs were obtained with a JEM 1230 electron microscope (JEOL, Japan).

Chloroplast and fragment proteins were electrophoretically separated in 12% polyacrylamide gel (pH 8.9) according to Laemmli [6]. Electrophoregrams were stained with organic stain Brilliant Blue R (Sigma, USA) and scanned; then band areas were calculated using the ScnImage program. For estimation of apparent molecular mass of separated proteins and their following identification, standard kits of marker proteins of Sigma were applied on electrophoregrams.

Chlorophyll (Chl) concentration was determined spectrophotometrically in 80% acetone extract as described by Arnon [7], and the Chl *a*/*b* ratio was determined according to Vernon [8]. Mean values of Chl *a*/*b* correspond to seven biological samples.

Absorption spectra at room temperature were registered with a Specord P200 spectrophotometer in digital mode in the range of 400-900 nm with a step of 1 nm. The cell thickness was 10 mm, chlorophyll concentration 10 μg/ml.

Spectra of low-temperature fluorescence were measured with a device assembled in our laboratory as described previously [9]. The spectra were measured in the range of 650-800 nm in digital mode with a step of 0.5 nm and 2 nm band-pass. The kinetics of P700 oxidation and dark reduction were measured at saturating actinic illumination on the device assembled in our laboratory as described previously [10].

RESULTS

Measurement of biochemical characteristics of the fragments has shown the following. The Chl *a*/*b* ratio in the fragments decreases on average by 19% compared with chloroplasts. Relative value of P700 signal at equal chlorophyll concentration in the chloroplast and fragment suspensions becomes 5 times lower for the latter (Fig. 1). Electrophoregrams of chloroplast and fragment proteins show a significant attenuation of CF, CP, and CP1 bands, the first of which corresponds to the ATPase complex protein, whereas the other two belong to proteins of PS I complex (Fig. 2). CP43, CP47 bands, and the band of oxygen evolving complex (OEC) of PS II are slightly attenuated on the electrophoregram of fragments, whereas the intensity of bands of LHC II zone shows practically no change. Area ratios of the same bands on electrophoregrams of the chloroplast and fragment proteins are shown in the table. Since equal volumes of suspensions with equal chlorophyll concentrations were used for electrophoretic separation of proteins, the above-mentioned ratios of band areas suggest changes in the content of proteins of supramolecular membrane complexes. It follows from the table that the content of PS I proteins in fragments decreases and the amount of ATPase proteins decreases even more. The content of LHC II proteins remains practically unchanged, and a slight tendency for decrease is observed for PSII proteins.

A shift to shorter wavelength of the red band maximum by 2 nm is found in the absorption spectrum of fragments along with decreased absorption in its long-wavelength part, as well as increased relative intensity in the Chl *b* absorption region at 650 nm (Fig. 3).

Low-temperature fluorescence spectra (Fig. 4) show both a significant decrease in relative intensity of the

Changes in contents of the main supramolecular membrane complex proteins in fragments compared with that in chloroplasts

Complex	Fragment/chloroplast*
PS I (CP1 + CP)	0.72 ± 0.04
PS II (CP43 + CP47 + OEC)	0.93 ± 0.09
CCK II (LHC II)	1.01 ± 0.04
ATPase (CF)	0.45 ± 0.05

* Band area ratio on electrophoregrams.

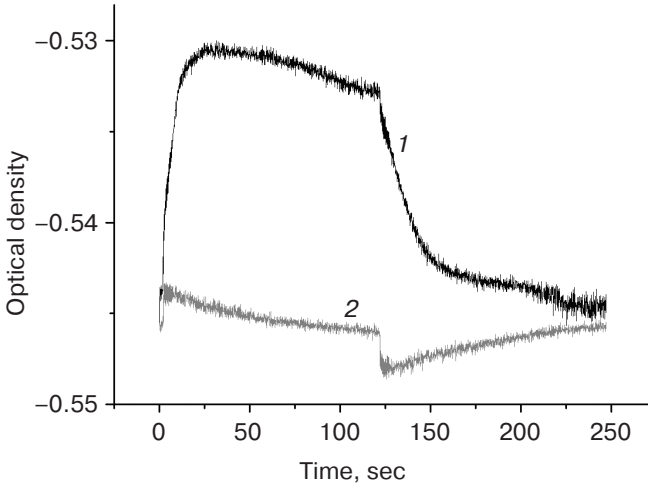

Fig. 1. Kinetic curves of P700 oxidation and dark reduction in suspensions of chloroplasts (*1*) and heavy fragments (*2*) (the curves are normalized to optical density at 700 nm).

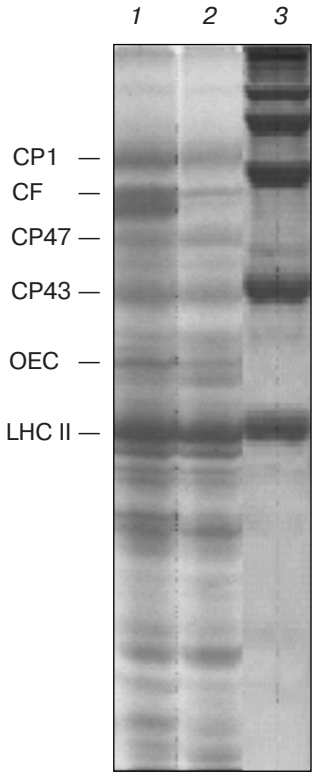

Fig. 2. Electrophoregrams of chloroplast (*1*) and heavy fragment (*2*) proteins and of a mixture of marker proteins (*3*).

long-wavelength band, corresponding to PS I emission, and its short-wavelength shift. Excitation spectra of long-wavelength fluorescence, shown in Fig. 5, were measured at equal chlorophyll concentrations in the chloroplast and fragment suspensions and normalized to equal fluorescence at 735 nm. In this case, a higher integral intensity in the range from 16 to 50% is revealed for fragments isolated from different batches of pea plants. The shape of the fluorescence excitation spectrum for fragments is changed compared with that for chloroplasts (Fig. 5).

Electron microphotographs of ultrathin sections of heavy-fragment preparations show these particles in longitudinal and cross sections (Fig. 6). Measurement of thylakoid diameter on these photographs gives the value of 410 ± 35 nm, and the amount of thylakoids on cross sections of the particles is in the range from 1 to 8. The distribution of particles with different thylakoid content is shown in Fig. 7. The mean value of the diameter of granal thylakoids, measured for chloroplasts from which the fragments were isolated, was equal to 450 ± 40 nm, the amount of thylakoid per grana varied from 6 to 20. It follows from the electron-microscopic data that fragments represent a population of grana reduced in the

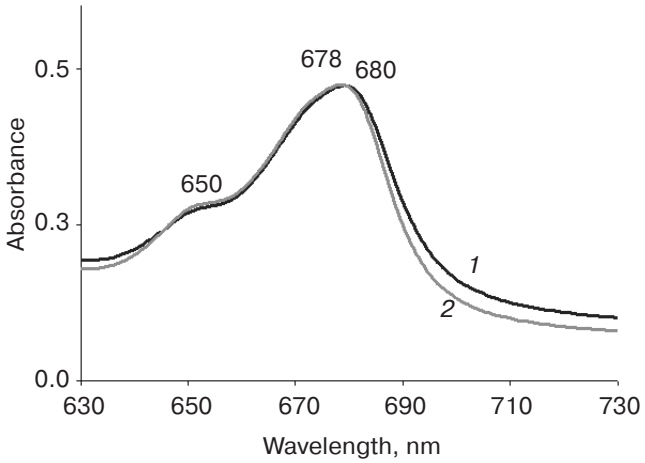

Fig. 3. Absorption spectra of chloroplasts (*1*) and heavy particles (*2*).

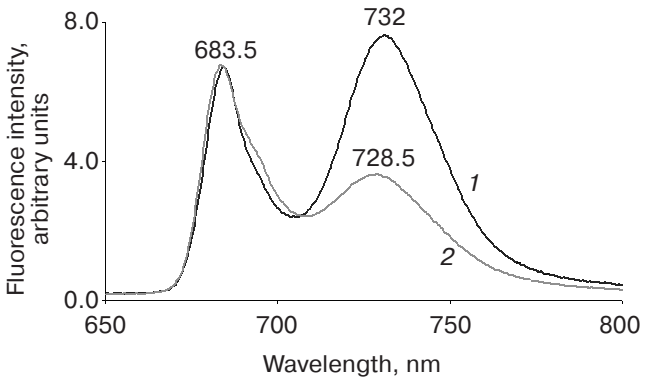

Fig. 4. Low-temperature fluorescence spectra of chloroplasts (*1*) and heavy fragments (*2*).

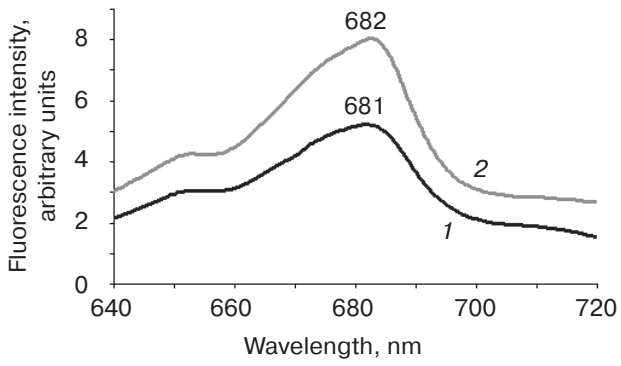

Fig. 5. Excitation spectra of fluorescence detected at 735 nm for chloroplasts (*1*) and heavy fragments (*2*).

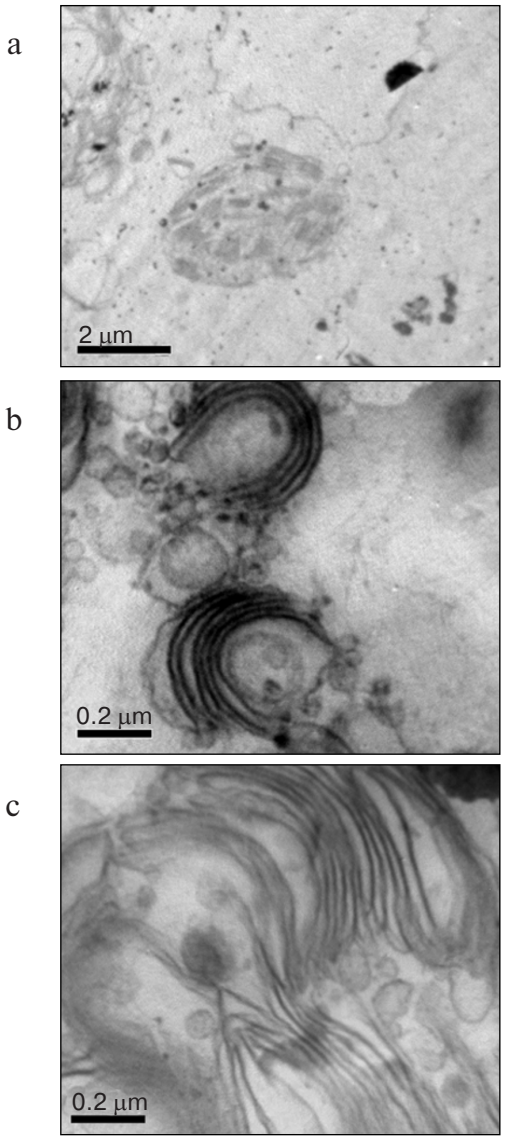

Fig. 6. Electron microphotographs of ultrathin sections of chloroplast (a) and heavy fragment fraction preparations (longitudinal section (b), cross section (c)).

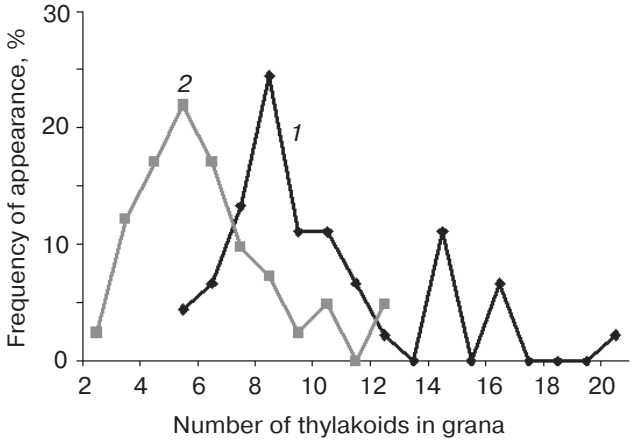

Fig. 7. Curves of grana distribution by thylakoid content for chloroplasts (*1*) and heavy fragments (*2*).

transversal direction. The diameter of thylakoids present in the fragments is close to that of granal thylakoids.

DISCUSSION

The results of measuring the biochemical characteristics have shown the lowered content of PS I complexes in the chloroplasts fragments. This follows from the decreased Chl *a/b* ratio, P700 signal, as well as from lowered contents of ATPase and PS I proteins (table and Fig. 2). Analysis of electrophoregrams shows that the depletion in the PS I complexes is due to those located in intergranal thylakoids. This follows from the lowered intensity of CF1 band, corresponding to the ATPase complex. As known, these proteins are localized on the surface of thylakoids that are in contact with stroma. Such are the intergranal thylakoids. This means that just this part of chloroplast ultrastructure is removed from heavy fragments.

The absorption and low-temperature fluorescence spectra also show the decrease in the content of PS I complexes in the fragments. Absorption is reduced in the long-wavelength part of red band in the spectrum, which is indicative of a lowered content of the long-wavelength chlorophyll forms, by which PS I complexes are enriched. A significantly decreased intensity in the long-wavelength band of the fluorescence spectrum caused by emission from PS I is observed. Thus, these specific alterations of biochemical and spectral characteristics support our previous idea [5] that intergranal thylakoids are substantially removed from heavy fragments.

Electron-microscopic investigation has shown that heavy fragments comprise a population of grana with decreased content of constituent thylakoids, the diameter of which is close to that in grana in the original chloroplasts.

The data show that the procedure of fragmentation makes possible the isolation of chloroplast grana, though with the decreased content of constituent thylakoids. Comparison of an average value of the thylakoid diameter within chloroplast grana and in isolated fragments shows no reliable difference between them, although there is a tendency to lowering of this parameter in the fragments. In this connection, the question arises concerning the extent of preservation of marginal regions of granal thylakoids. It is seen in the table that the LHC II content in the fragments is practically the same as in chloroplasts and the decrease in the content of PS II proteins is rather small. This suggests a sufficiently high extent of preservation of the granal thylakoid integrity. Proteins of the LHC II complex are present only in granal thylakoids, both in central and marginal regions. The PS II complexes are also localized mainly in the above-mentioned regions of granal thylakoids with the exception of their small amount (about 4% according to Albertsson [11, 12]) present in intergranal thylakoids. This estimation coincides with observation of slightly lower content of PS II complexes in our heavy fragments (table).

It follows from the table that the fragments contain about 70% of PS I proteins of their total amount in whole chloroplasts. This approximately 1.5 times exceeds Albertsson's estimation on the chlorophyll basis [12]. According to his model, grana contain only about 45% of PS I proteins, belonging to PS Iα, located in marginal region of granal thylakoids. However, in our case such a discrepancy points to a higher integrity of granal thylakoid rather than to its reduction. It should be also noted that according to our data PS I complexes are present in the central region of granal thylakoids as well [13], which can increase their total content in granal thylakoids compared to Albertsson's model that suggests their location in marginal regions only. Owing to this, we believe that the data on the estimation of PS I protein contents in our fragments agree with the concept of a high extent of preservation of marginal regions in thylakoids, present in the fragment fraction, and confirm our supposition about their presence in central regions [13]. It should be noted that the comparison of excitation spectra of long-wavelength fluorescence of chloroplasts and fragments supports our previous supposition about a larger light-harvesting PS I antenna in granal thylakoids [13]. This follows from the observation of a higher integral intensity of excitation spectrum of long-wavelength fluorescence, which under the conditions of our measurement is indicative of a larger value of absorption section for PS I in granal thylakoids.

Our fragments retain about 45% of ATPase proteins, which should point to essential preservation of the marginal region. In fact, the above-mentioned complexes are localized in grana mainly on the surface of marginal regions as well as on "end" membranes of grana. As shown for wheat chloroplasts [2], the ratio of the length of the marginal region membranes of granal thylakoids and intergranal thylakoids is 1 : 1.47. In this case, marginal regions should contain about 40% of ATPase complex if it is evenly spread over these surfaces.

The shape of marginal regions of granal thylakoids in ultrathin sections of fragment preparations is similar to those on chloroplast pictures (Fig. 6). The specific form of margins of each thylakoid and displaying of the destacking region are indicative of preservation of membrane components providing for specific shape of the thylakoid marginal regions. The comparison of properties of granal thylakoids present in fraction B3, obtained by Albertsson et al. [4] with chloroplast sonication, reveals the similarity with granal thylakoids in our heavy fragments. Particles of B3 fraction are characterized by a lowered Chl *a/b* ratio relative to P700 content, as well as by a decreased content of PS I and ATPase complexes. Similarly to our particles, a larger size of the PS I light-harvesting antenna was registered. The yield of heavy fragments in our case was in the range of 17-30% in different experiments, which is close to the level of B3 yield of 37% obtained by Albertsson [4]. Thus, the comparison of characteristics of our heavy fragments with those of B3 fraction in [4] suggests that in both cases granal thylakoids, present in fractions, retain the integrity of marginal regions to a certain extent.

In our case special interest is attracted to the fact that in the fragments, along with extremely weak reduction of granal thylakoids in the lateral direction, there is more significant reduction in the transversal direction. We suggest a possible cause of this on the basis of our hypothetical model of grana organization [14]. According to this model, grana contain fragments incorporating 3-4 thylakoids, separated by so-called "non-typical" granal thylakoids. The latter differ in composition from "typical" grana thylakoids [15] and are bound to them more weakly. According to our hypotheses, "non-typical" granal thylakoids are the continuation of intergranal thylakoids into grana [15]. Thus, they probably are the regions within grana which are removed by digitonin treatment that caused the grana separation into fragments, each of which contains several more strongly bound "typical" granal thylakoids.

REFERENCES

1. Rozak, P. R., Seiser, R. M., Wacholtz, W. F., and Wise, R. R. (2002) *Plant Cell Environ.*, **25**, 421-429.
2. Sharkova, V. E., and Bubolo, L. S. (1996) *Fiziol. Rast.*, **43**, 409-417.
3. Wollenberger, L., Stefansson, H., Yu, S.-G., and Albertsson, P.-A. (1994) *Biochim. Biophys. Acta*, **1184**, 93-102.
4. Andreasson, E., Svensson, P., Weibull, C., and Albertsson, P.-A. (1988) *Biochim. Biophys. Acta*, **936**, 339-350.

5. Kochubey, S. M., Shevchenko, V. V., and Bondarenko, O. Yu. (2005) *Dokl. NAN Ukr.*, **4**, 161-166.
6. Laemmli, U. K. (1970) *Nature*, **292**, 200-202.
7. Arnon, D. I. (1949) *Plant. Physiol.*, **24**, 1-15.
8. Vernon, L. R. (1960) *Anal. Chem.*, **32**, 1144-1150.
9. Volovik, O. I., Korneev, D. Yu., Porubleva, L. V., and Shevchenko, V. V. (2003) *Fiziol. Rast.*, **50**, 325-331.
10. Kochubey, S. M., Vovk, A. I., Bondarenko, O. Yu., Shevchenko, V. V., Bugas, R. V., Mel'nik, A. K., and Tanchuk, V. Yu. (2007) *Biochemistry (Moscow)*, **72**, 558-564.
11. Albertsson, P.-A. (1995) *Photosynth. Res.*, **46**, 141-149.
12. Albertsson, P.-A. (2001) *Trends Plant Sci.*, **6**, 349-354.
13. Kochubey, S. M., Shevchenko, V. V., and Bondarenko, O. Yu. (2003) *Fiziol. Rast.*, **50**, 325-331.
14. Kochubey, S. M., Shevchenko, V. V., and Korneev, D. Yu. (2007) *Structural Organization and Functional Peculiarities of the Light Phase of Photosynthesis* [in Russian], Logos, Kiiv.
15. Kochubey, S. M., Shevchenko, V. V., and Bondarenko, O. Yu. (2005) *Fiziol. Rast.*, **52**, 499-506.