2-D gel densitometer for high-contrast and selective imaging of chlorophyll-containing protein complexes separated by non-denaturing polyacrylamide gel electrophoresis

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Abstract

In this work, we present a home-made two-dimensional (2-D) CCD imaging system for the monochromatic densitometry of plane gels and its application to the imaging and densitometry of chlorophyll (Chl)-containing proteins separated by non-denaturing polyacrylamide gel electrophoresis. The monochromatic imaging of separated green bands at the wavelengths corresponding to their absorption band increases their contrast. This allows a better visualization of the faint-green bands in the gel and using of samples with lower Chl content for the electrophoresis. By the comparison of 2-D densitograms of the same gel illuminated with 670 and 650 nm lights, that is, at the red absorption maximum of Chl $a$ and $b$, respectively, we achieved a selective imaging of the complexes with different Chl $a/b$ ratio. This approach was used to specify an unknown band that appeared in the gel of the sample prepared from the thylakoid membranes of preheated barley leaves.

Keywords: CCD densitometry; PAGE; Chlorophyll $a/b$ ratio; Light-harvesting complex; Photosystem core

1. Introduction

The chlorophyll (Chl)-containing protein complexes from thylakoid membranes of chloroplasts are frequently separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) using mild detergents (see e.g. Ref. [1]). Usually, several samples in sepa-
rated lines are electrophoresed at once. The result of this technique are lines with several
green or yellow-green bands in the transparent plane gel. To display the results, this gel is
often photographed in white light with a color or black and white camera [2]. However, the
faint-green bands are visible barely in resulting photographs (e.g. Refs. [1,3]). For a better
resolution of separated bands in the individual lines, densitometric measurements using a
narrow beam of monochromatic light corresponding to red absorption maximum of Chl
molecules (670 and 650 nm, for Chl \( a \) and \( b \), respectively) are used (e.g. Refs. [4,5]). The
ratio of the resulting optical densities of the band obtained at 670 and 650 nm lights can be
used for a rough estimation of the Chl \( a/b \) ratio in the green band. Such a scanning is
performed using beam densitometers that are often supplied as accessory equipment to
spectrophotometers.

Recent sensitive CCD cameras allow taking densitograms of the whole plane gel within
a fraction of a second. However, commercial CCD imaging systems use mostly white or
broad-band light scanning for densitometry measurements and are not constructed for
densitometry at monochromatic light. In this work, we describe our cheap home-made
CCD imaging system for monochromatic densitometry of plane gels and its application for
high-contrast imaging of Chl-containing proteins separated by mild native PAGE.

2. Materials and methods

2.1. Plant material

*Hordeum vulgare* L. (cv. Akcent) was grown in a growth chamber at 25 °C on the
artificial soil composed of perlit and Knop solution. The light regime was 16 h light (90
\( \mu \text{mol m}^{-2} \text{s}^{-1} \) of PAR)/8 h dark. Detached primary leaf segments of 8- to 9-day-old
barley seedlings were used for preparation of thylakoid membranes and subsequent
electrophoresis.

2.2. Non-denaturing Deriphat-PAGE

Our procedure used for non-denaturing Deriphat-PAGE separating Chl-containing
protein complexes was a slight modification of the procedures described by Peter and
Thornber [1,6] and Peter et al. [7]. Thylakoid membranes were isolated from control
unheated or preheated (70 °C, 10 min) barley leaves. The leaf segments were mixed with
chilled grinding buffer (0.4 M sorbitol, 10 mM Tricine–NaOH, pH 7.6, 10 mM MgCl\(_2\))
and homogenized three times for 5 s (24,000 revolutions per minute) by a homogenizer T
25 basic (IKA Labortechnik, Staufen, Germany). The homogenate was filtered through
four layers of miracloth and centrifuged at 3020 \( \times g \) for 2 min at 4 °C. The chloroplast
pellet was resuspended in 1 ml of lysis buffer (25 mM Tricine–NaOH, pH 7.6, 2 mM
Na\(_2\)EDTA) and the suspension was centrifuged at 22,000 \( \times g \) for 5 min at 4 °C. The
obtained thylakoids were resuspended and diluted to the final concentration 1 mg Chl
\( a+b/\text{ml} \) in the extraction buffer containing 11.3 mM Tris, 87 mM glycine and 9% v/v
glycerol. The Chl \( a+b \) content was determined spectrophotometrically in 80% acetone
according to Ref. [8]. The thylakoid membranes were also diluted in the extraction buffer
to obtain 1/2, 1/3, and 1/4 of the final concentration for the control unheated leaves and to 1/3 of the final concentration for the preheated leaves.

The membrane solubilization was carried out with 20% decyl maltoside. This surfactant and membranes were mixed to yield a final 20:1 (w/w) ratio of surfactant to Chls. The surfactant extracts were centrifuged at 7000 g for 2 min at 4 °C to remove the colorless insoluble material, and the green supernatant was immediately applied to PAGE.

A polyacrylamide gel of 8.5% [33.5% (w/v) acrylamide, 0.3% (w/v) bisacrylamide] containing 12.4 mM Tris, 48 mM glycine, pH 8.6, was polymerized with 0.1% ammonium persulfate, Na2SO3 (1.5 mg per 1 ml of gel) and 0.005% TEMED. For the 3-mm-thick gel,
a volume of 13 μl of extract was loaded per line. The cooled reservoir buffer for electrophoresis was 12.4 mM Tris, 96 mM glycine, pH 8.3, and 0.2% Deriphat 160. The gel was electrophoresed (at 4 °C) at 50 V constant voltage for 15 min and then at 90 V for 120 min (a device of Bio-Rad, USA) in darkness.

2.3. CCD imaging of Chl-containing proteins

The imaging system consists of a halogen lamp (Osram, 150 W), condenser, interference filter (650 or 670 nm, bandwidth 10 nm), beam integrator, pinhole, collimating lens, disc diffuser, objective and a cheap greyscale CCD camera OS-45D (Oscar, Taiwan) with switched off gamma correction. The plane gel with several lines was placed between the objective and diffuser (see Fig. 1). Video signal from the camera was grabbed and processed with an Aver-EZCapture video card (AverMedia, Taiwan) plugged into a PC. Each pixel of the greyscale image of the whole gel had the value from 0 (black) to 255 (white). The spectral sensitivity of the whole CCD imaging system was approximately the same for 650 and 670 nm. A linearity of CCD response to changing light intensity for both wavelengths was verified. Heterogeneity of light on the level of gel surface did not exceed 5%. All densitograms were corrected for the light heterogeneity profile pixel by pixel.

To reduce the noise of detected densitograms, they were grabbed as 5s “AVI” movies consisting of 21 frames without any compression. These movies were sampled to individual frames using Corel Photo-Paint 8 (Corel, USA) and these frames were averaged using the software Matlab 5.2 (Mathworks, USA). Other mathematical operations with images (subtraction, summation of grey levels of pixels in rows) were also performed with Matlab 5.2. supported by Excel 97 (Microsoft, USA). A conversion of grey levels of pixels to artificial colors was performed with Origin 5.0 (Microcal software, USA).

3. Results and discussion

3.1. Construction of two-dimensional (2-D) gel imaging apparatus for monochromatic densitometry

We constructed a cheap imaging apparatus that can be used, in general, for a monochromatic densitometry of plane samples. White light emitted from the halogen lamp is filtered through the interference filter before the sample to avoid a possible photodestruction of sample substances. Homogeneity of the monochromatic light used for illumination of the sample surface was achieved by the beam homogenizer and disc diffuser (see Fig. 1; for other details, see Materials and Methods). This apparatus was used for the densitometry of Chl-containing protein complexes separated by native Deriphat-PAGE according to Peter and Thornber [1,6] and Peter et al. [7].

3.2. High-contrast imaging of green bands in the gel

The first line in Fig. 2 [W(C)] shows a color picture of the electrophoreogram with Chl-containing protein complexes taken by a sensitive digital camera Olympus C-3000 in
transmitting white light. The off mode flash and white balance for a tungsten lamp was
used during picture taking. While PSI, LHCt, LHCm, and FP bands representing Photo-
system I, trimeric and monomeric light-harvesting complexes, and free pigments are
visible, CCI and CCII bands representing core complexes of Photosystems I and II,
respectively, are very faint. The relatively low content of the photosystem core complexes
was expected as the used samples were prepared from barley leaves grown at low light [9].
The picture of this electrophoreogram is similar to the picture reported in Ref. [3]. The Chl
concentration (1 mg Chl $a+b$/ml) and sample volume (13 $\mu$l) loaded into the line was the
same as those used in Ref. [3].

The same line was also scanned by our imaging apparatus with a black and white
digital camera using white light illumination (without interference filter) and with 670 nm
monochromatic light [Fig. 2, denoted as W(BW) and M 1, respectively]. While CCII and
CCI bands were almost invisible in white light, similarly to the color picture [W(C)], there
was much more contrast in 670 nm light that corresponds spectrally to the red absorption
maximum of Chl $a$ in Chl–protein complexes. It is not very surprising because in general,
the imaging of color objects in white light reduces their contrast compared to the imaging
with colors corresponding spectrally to the absorption bands of the color object. This
reduction of contrast is pronounced for the objects with narrow absorption bands like those
of Chl molecules.

The high contrast of the separated bands in 670 nm light enables the use of lower Chl
amounts in the sample. The three lines on the right side of Fig. 2 (M 1/2, M 1/3 and M 1/4)
represent electrophoreograms of the same sample as M 1 but with 1/2, 1/3 and 1/4 Chl
content per line, respectively. These diluted samples were prepared in the following way.
Isolated thylakoid membranes were firstly diluted to the required Chl concentration and
then the surfactant (decyl maltoside) was added to ensure that the surfactant to Chls had

![Figure 2](image-url)
the same weight ratio—20:1—as recommended in the procedure of Peter and Thornber (e.g. Ref. [6], see also Materials and Methods). Then the same sample volumes as for M 1 sample (13 μl) were loaded into the gel lines. Electrophoreograms of these samples were scanned in the same way as M 1. Apart from a reduction of each band caused by the lower Chl concentration in the sample, it is visible that a relatively pronounced LHCm band in M 1 sample practically disappeared for M 1/3 and M 1/4 samples.

To explain these relative changes in the LHCm amount in the electrophoreograms, we also made the electrophoreograms of the samples having a constant volume concentration of the surfactant (the value was as for M 1 in the previous experiment) and thereby a different surfactant/Chl ratio. In contrast to the previous experiment, we observed that with decreasing Chl concentration in the sample, all the bands in electrophoreograms diminished proportionally (data not shown). As we did not observe any green band on the top of the gel in both experiments, we suppose that the Chl-containing protein complexes were always fully solubilized. The results show that although the surfactant/Chl ratio is unchanged, the surfactant concentration in the sample influences qualitatively the solubilization of native Chl-containing protein complexes. This indicates that in our case, the LHCm band, which was pronounced in the samples with higher volume concentration of the surfactant, could be, at least partly, a solubilization artifact and that LHCt mainly represents the native state of LHCs. This problem deserves a separate study.

3.3. Calculation of one-dimensional (1-D) densitograms

Fig. 3 shows 1-D densitograms of M 1–M 1/4 samples that are usually measured using beam densitometers. In our case, the densitograms were calculated from the images shown in Fig. 2 by averaging grey levels of pixels in the rows of each line and a conversion to absorbance units by logarithmic transformation, since the raw grey levels of image pixels correspond to the gel transmittance. These densitograms were multiplied by a concentration factor, that is, 1, 2, 3, and 4, for M 1, M 1/2, M 1/3 and M 1/4, respectively. If the

![Fig. 3. Densitograms evaluated from the electrophoreograms obtained in monochromatic 670 nm light. 1, 1/2, 1/3 and 1/4 denote relative Chl concentrations in the sample loaded into the individual lines. For other details, see the legend of Fig. 2 and the text.](image-url)
amount of Chl-containing proteins in the band reflected the sample concentration only, then all four densitograms would agree. Fig. 3 clearly shows that, as it was mentioned above, decreasing surfactant concentration in the sample leads to a gradual relative decrease of LHCm in the electrophoreogram. Concomitantly, the band with free pigments (FP) appears at lower migration distances. The appearance of relatively lower LHCt and PSI bands, both containing LHCm complexes, for the samples with higher surfactant and Chl concentrations (M 1 and M 1/2) agrees with the suggested increase in aggressivity of surfactant action as discussed above.

3.4. Selective imaging of separated Chl-containing protein complexes

Separated Chl-containing protein complexes differ in the Chl $a/b$ ratio. Chl $a$ molecules in protein complexes absorb mainly at about 670 nm in the red spectral region while Chl $b$ absorbs mainly at about 650 nm. However, the red absorption bands of both Chls overlap. Nevertheless, the ratio of absorbances of separated bands in the gel at both wavelengths can be, in principle, used for a rough differentiation of separated Chl-containing protein complexes in the gel. This can be helpful mainly in the case of the appearance of an unknown band in the gel.

Using our 2-D densitometric system, we tried to identify the unknown band that appeared in the electrophoreogram of the sample prepared from thylakoid membranes of barley leaves preheated for 5 min at 70 °C. The first line on the left side of Fig. 4 shows electrophoreogram with Chl-containing protein complexes from thylakoid membranes of untreated barley leaves illuminated with 670 nm light (the same picture is in Fig. 2 denoted as M 1/3). The second line represents the electrophoreogram of the sample prepared from

Fig. 4. Electrophoreograms with separated Chl-containing proteins from thylakoid membranes of control unheated (C) barley leaf segments (see also Fig. 1) and leaf segments preheated for 10 min at 70 °C (H). Left pair are electrophoreograms scanned with 670 nm light. Middle and right pairs are the same samples displayed with ratio type of imaging (grey level of each pixel of densitogram scanned with 650 nm light divided by that of the densitogram scanned with 670 nm light and multiplied by 255—range of greyscale levels) using greyscale levels and artificial colors, respectively. For detailed description, see the text.
preheated barley leaves with the same Chl concentration as in the first line. Both electrophoreograms were also illuminated with 650 nm light (not shown). For the selective visualization of bands in the gel, we transformed the grey levels of each image pixel of both electrophoreograms to the absorbance scale. Then we divided the level of each pixel in the 650 nm image by that of the 670 nm image and this ratio multiplied by 255 (the range of greyscale levels, 0—black, 255—white). The result of these operations is shown in the second pair of lines in Fig. 4 [denoted as ratio(BW)]. The lighter bands represent Chl-containing protein complexes with higher absorbance ratio A650/A670 indicating their lower Chl a/b ratio. As it can be expected, the bands containing trimeric light-harvesting complexes of Photosystem II (LHCt) with low Chl a/b ratio are lighter than the other bands in this line.

Looking at the electrophoreograms of these two samples obtained in 670 nm light, it is evident that a dense new band appearing in the preheated sample at the position of CCII represents either some aggregated LHCs or disintegrated PSI. As this new band is relatively dark in the ratio type imaging [see the ratio(BW) in Fig. 4], the latter is more probable. We visualized the differences of grey levels of each pixel by assigning artificial colors [Fig. 4, denoted as the ratio(color)]. It is clearly visible that the new band has Chl a/b ratio similar to that of PSI. Thus, the new band represents some disintegrated form of PSI as was also confirmed by low-temperature fluorescence spectroscopy (results not shown).

4. Simplified description of the method and its (future) applications

The presented 2-D densitometric system is based on a digital black and white CCD camera and allows to measure densitograms of plane gels in monochromatic light separated by a suitable interference filter. Generally, this system can be used for imaging of any color component with higher contrast. We used this system for a high-contrast imaging of Chl-containing protein complexes separated by native PAGE. This approach allows to use samples with lower Chl amounts. Due to the fact that the complexes separated by the native PAGE contain different amounts of Chl a and Chl b and their absorption bands in the red spectral region slightly differ, this densitometric system can be also used for a selective imaging of the separated Chl-containing protein complexes.

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References


