

# Digitonin Precipitable Sterols Inducing the Aggregation of Prolamellar Body-Like Structures from Completely Dissolved PLB Components

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Z. Naturforsch. **37 c**, 251–255 (1982); received December 16, 1981

Prolamellar Body, Reaggregation, Sterols

Completely dissolved lipids of a prolamellar body/primary thylakoid (PLB/PT) fraction from etiolated *Avena sativa* plastids were reaggregated *in vitro*. The analysis of the originating tubular structures showed a qualitative identity in their lipid content with that of the native PLB/PT fraction. Reaggregation experiments also showed that not all lipids constituting the PLB are necessary to build up the tubular structures. Only avenacosids A and B together with 3- $\beta$ -hydroxy-sterols are the obligatory structural components.

## Introduction

Completely dissolved components of prolamellar bodies (PLB) from etioplasts of *Avena sativa* reaggregate into tubules resembling those of native PLB [1]. Ruppel *et al.* [2] showed that proteins had no influence on the reaggregation of PLB so that only lipids and/or pigments must be responsible for the tubular structures.

Using this phenomenon of reaggregation it is possible to investigate the PLB building units by isolating and analysing reaggregated tubules. This experimental system will be preferred over the analysis of native PLB because the separation of primary thylakoids (PT) from usually interconnected PLB creates no problem.

Reaggregation experiments also allow to show which component of the PLB is necessary to build up the tubular structure and which additional components do complete the PLB.

## Materials and Methods

### a) Plant material

*Avena sativa* L. germinated at 25 °C and 75% humidity in complete darkness.

**Abbreviations:** DGDG, digalactosyl diglyceride; EM, electron microscope; MGDG, monogalactosyl diglyceride; PCHLIDE, protochlorophyllide; PLB, prolamellar body; PT, primary thylakoid; SQDG, sulfoquinovosyl diglyceride; S, solvent system; TLC, thin-layer chromatography.

### b) Isolation of PLB/PT

500 g of 6–7 days old seedlings of *Avena sativa* were harvested under green safety light and homogenized in 1.5 l 0.05 M Tris/HCl buffer pH 8.0, containing 0.25 M sucrose. After filtering through 8 layers of cheesecloth the suspension was centrifuged 20 min at 1500  $\times$  g. The etioplasts were shocked osmotically by resuspending them in 50% Percoll (Percoll/0.05 M Tris/HCl pH 8.0). After formation of the gradient by centrifugation (15 min at 10 000  $\times$  g) PLB and PT banded at a density of 1.06 g/ml (control by EM). The isolated PLB/PT fraction was washed and centrifuged three times with 0.05 M Tris/HCl buffer pH 8.0 (15 min 5000  $\times$  g).

### c) Extraction of lipids, saponins and pigments.

The isolated PLB/PT fraction was extracted with 80% acetone. Precipitated proteins and the rest of Percoll were removed by centrifugation (15 min 5000  $\times$  g).

### d) Thin-layer chromatography (TLC)

Extracts were chromatographed on Silgur 25 (Machery & Nagel) and on HPTLC-precoated plates (Silica Gel 60 with concentrating zone, Merck) in the following solvent systems (S 1–3):

S 1	CHCl <sub>3</sub> : MeOH : H <sub>2</sub> O	= 70 : 30 : 4
S 2	CHCl <sub>3</sub> : MeOH : H <sub>2</sub> O	= 140 : 30 : 4
S 3	petroleumether : diethylether	= 5 : 1

After TLC in the different solvent systems, components were identified by spraying the plates only

partially with 1% orcinol in 2N  $\text{H}_2\text{SO}_4$  or with 1% anisaldehyde in acetic acid:  $\text{H}_2\text{SO}_4 = 50:1$  or compounds were located with iodine vapour. The corresponding untreated spots were scraped off and lipids were eluted with 80% acetone.

#### e) Reaggregation experiments

The complete acetone extract and its TLC-isolated components and cholesterol and  $\beta$ -sitosterol (both as standards from Boehringer) were tested for reaggregation. All lipids were dissolved in 80% acetone and brought to reaggregation by evaporation of the solvent (40–50 °C). The process of reaggregation was controlled by electron microscopy.

#### f) Isolation of reaggregation products

1 ml of the suspension containing tubules was mixed with 10 ml of 25% Percoll (in Tris/HCl 0.05 M pH 8.0). After the formation of the self-generating gradient (30 min at 20 000  $\times g$ ), tubules banding at a density of 1.04 g/ml could be isolated.

#### g) Precipitation of the reaggregation factor (R-factor) by digitonin

After addition of 1 volume of 1% digitonin in 80% acetone the acetone extract was centrifuged for 15 min at 5000  $\times g$ . The pellet was dissolved in solvent system 1.

#### h) Electron microscopy

After negative staining with 1% Na-phosphotungstic acid (pH 7.0) all preparations were examined on a Hitachi H-500 electron microscope.

## Results and Discussion

#### a) Lipid composition of native PLB/PT and reaggregated tubules

To find out the composition of PLB and its building units the isolated PLB/PT fraction was extracted with acetone. The lipid composition of native PLB/PT could be determined by TLC using solvent system 1 (Fig. 1, II): saponins (A and B), MGDG, DGDG, SQDG, sterol glycosides and pigments were found to be the main constituents. These results agree with the lipid composition of native PLB/PT found by other authors [3–5].

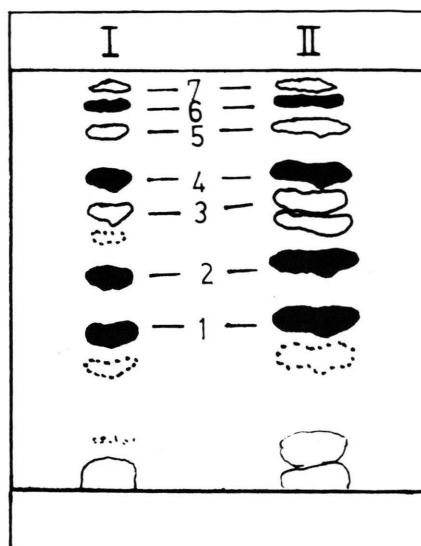


Fig. 1. Thin-layer chromatogram of the acetone extract of isolated reaggregated tubules (I) and of isolated PLB/PT of 6 days old seedlings from *Avena sativa* (II) (solvent system 1, stain: 1% orcinol in 2N  $\text{H}_2\text{SO}_4$ )

spot 1: avenacosid B; spot 2: avenacosid A; spot 3: SQDG; spot 4: DGDG; spot 5: sterol glycoside; spot 6: MGDG; spot 7: pigments.

Assuming that only lipids constituting native PLB are capable of reaggregation, extracts of PLB/PT were used for reaggregation experiments. The lipid content of the reaggregation products was then compared to that of the complete PLB/PT-extract. Therefore lipids of the acetone extract of PLB/PT were allowed to reaggregate: the resulting yellow tubules banded after centrifugation in 25% Percoll at a density of 1.04 g/ml (Fig. 2). These tubules showed an intense red fluorescence probably due to PCHLIDE. The absorption spectra of the pigment extract from reaggregated tubules confirmed the presence of PCHLIDE and carotenoids.

After TLC(S 1) the comparison between the lipid composition of the reaggregated tubules and that of the complete acetone extract of PLB/PT did not show differences (Fig. 1, I and II). Reaggregated tubules consisted mainly of avenacosid A and B, MGDG, DGDG, PCHLIDE and carotenoids, as did the native material. There are two possible interpretations of these results:

- PLBs are qualitatively identical to PT but might be quantitatively different.
- PLBs appear to be identical to PT as components of native PTs in addition are incorporated *in vitro*.

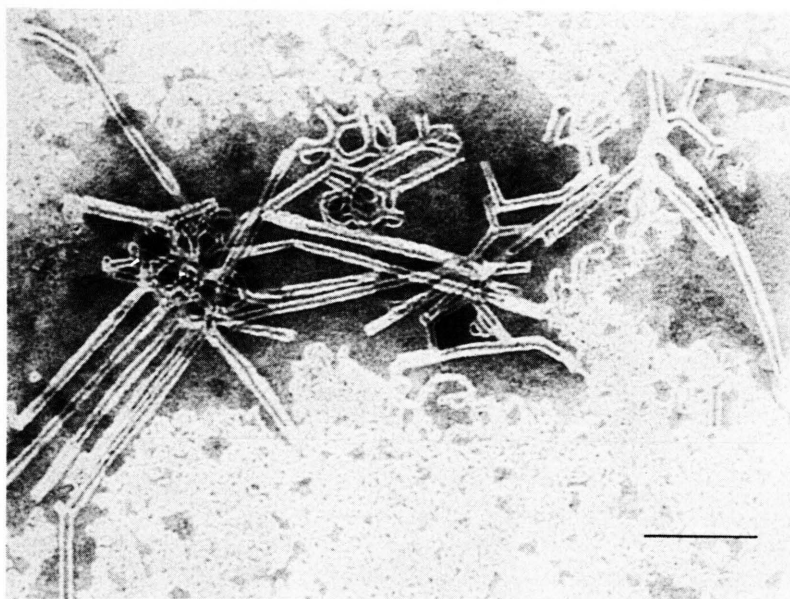


Fig. 2. Reaggregated tubules built up by complete acetone extract of isolated PLB/PT, seen in the electron microscope after negative staining (bar: 0.2  $\mu$ m).

A loose association of some lipids to the surface of reaggregated tubules can be excluded because rigorous handling of tubules like centrifugation and washing did not cause a loss of any component.

*b) Characterization of components necessary for reaggregation of tubular structures*

First the role of avenacosids was investigated as they are assumed to be the main building units of the PLB [4]. By means of TLC(S1) the 2 saponins were isolated from the acetone extract of PLB/PT. Fraction A (avenacosids) and fraction B (acetone extract minus avenacosids) were tested for their ability to build PLB-like tubules *in vitro*. Neither fraction A nor fraction B alone formed reaggregated tubules. Only both fractions together allowed the PLB components to reaggregate into tubules.

To find out which components of fraction B were necessary for the reaggregation of tubules – in addition to fraction A (saponins) – the fraction B was split up after TLC(S1). The chromatogram was divided into different zones and each zone was tested to reaggregate with the saponins. Only the components with a  $R_f$  value greater than that of MGDG(TLC, S1, Fig. 1) were able to reaggregate with saponins into tubular structures. These components are therefore called reaggregation factor (R-factor).

By variation of the TLC solvent system (S2) the unknown R-factor could be characterized as two closely related, orcinol- and anisaldehyde positive spots (Fig. 3). Their positive reaction with a specific stain for sterols and terpenes gave a preliminary idea how to classify the R-factor chemically.

After changing the TLC-solvent system from chloroform-methanol-water to petroleumether-di-

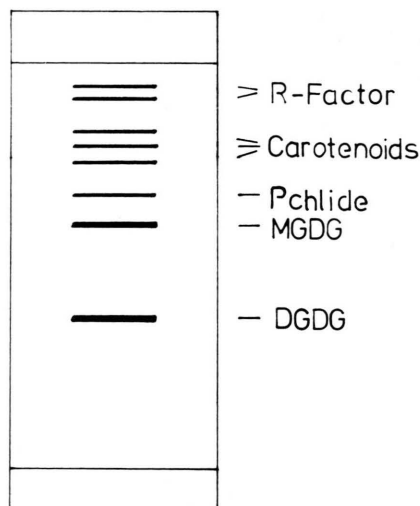


Fig. 3. Thin-layer chromatogram of the acetone extract of isolated PLB/PT in solvent system 2.  
R-factor: reaggregation factor.

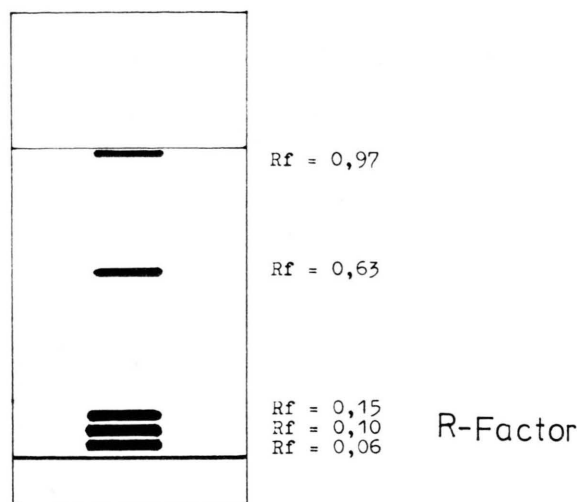


Fig. 4. Thin-layer chromatogram (solvent system 3) of the reaggregation factor (R-factor) isolated by solvent system 2.

ethylether (S3) the R-factor splitted into 5 spots (Fig. 4): a triplet of components near the start and 2 spots with greater  $R_f$  values. Only the triplet of components cooperated with the saponins to reaggregate into tubular structures. The lowest spot of the triplet had the same  $R_f$  value as cholesterol and  $\beta$ -sitosterol, run as standards.

To give more details on the triplet of components absorption spectra from each of the 3 spots were car-

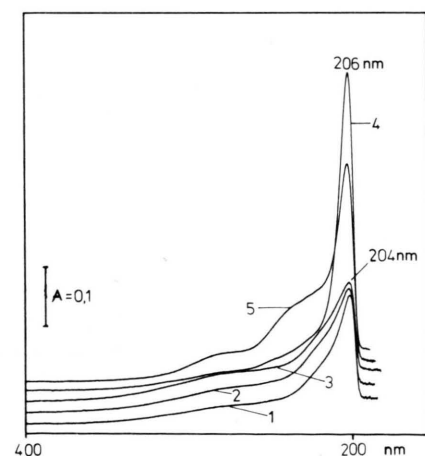


Fig. 5. Absorption spectra of the components of the R-factor and of known sterols in methanol ( $A$  = absorbance): R-factor component with  $R_f$  value 0.06 (S3) = 1; R-factor component with  $R_f$  value 0.1 (S3) = 2; R-factor component with  $R_f$  value 0.15 (S3) = 3;  $\beta$ -sitosterol = 4; cholesterol = 5.

ried out which revealed maxima at 204 nm resembling the spectra of cholesterol and  $\beta$ -sitosterol (Fig. 5). These findings support the assumption that the R-factor consists of sterol-like substances. To confirm this hypothesis experiments were performed for testing the ability of sterols to reaggregate with saponins instead of the R-factor. Fig. 6 and Table I show that 3- $\beta$ -hydroxy-sterols in cooperation with saponins built up the same PLB-like structures as the R-factor did.

If the R-factor belongs to the family of 3- $\beta$ -hydroxy-sterols like cholesterol it has to precipitate with digitonin. Therefore the PLB/PT acetone extract was treated with digitonin. After centrifugation the supernatant was free of R-factor whereas the pellet consisted of the triplet of components of the R-factor mentioned above (controlled by TLC, S3). According to our hypothesis it was impossible to find reaggregated tubules within the supernatant because the R-factor (e.g. 3- $\beta$ -hydroxy sterols) precipitated by digitonin was lacking (Table I). Therefore tubules are supposed to be built up by forming a complex of saponin with 3- $\beta$  hydroxy sterols.

As both components of the complex (tubules) are proved to be part of the plastids [4, 6–8], we tend to translate the results *in vitro* to the *in vivo* situation: the saponins as well as the 3- $\beta$ -hydroxy-sterols are necessary structural components of tubules. It is possible that they may integrate other lipids found in PLBs.

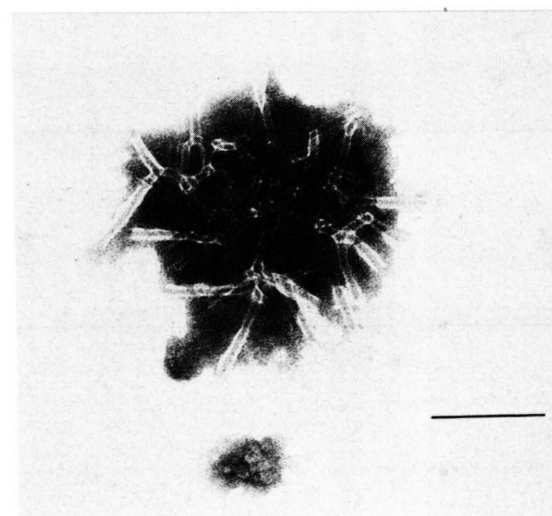


Fig. 6. Reaggregated tubules, built up by purified saponins A and B and cholesterol, seen in the electron microscope after negative staining (bar: 0.2  $\mu$ m).

Table I. Reaggregation experiments with components of the acetone extract of PLB/PT and with some sterols in combination with saponins.

	Saponins (A and B)	Acetone extract (saponins included)	Acetone extract (minus saponins)	Acetone extract (minus R-factor)	R-Factor	Cholesterol	$\beta$ -Sitosterol
reaggregation of itself	–	+	–	–	–	–	–
reaggregation with saponins			+	–	+	+	+

+: reaggregated tubules.

–: no reaggregated tubules.

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