The Determination of Prolamellar Bodies and Saponins in Etioplasts and Leaves of *Avena sativa* L.

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Z. Naturforsch. **35 c,** 519–521 (1980);
received January 21, 1980

Etioplasts, Prolamellar Bodies, Saponins, Chromatography

Prolamellar body tubules in etioplasts are composed mainly of two saponins. The determination of these saponins in leaves, etioplasts or subfractions of etioplasts is recommended to evaluate the concentration of these tubules in a given fraction. Using a densitometer after TLC-separation of the saponins measurements are carried out in a short time with high accurancy. Also the absolute concentration of these saponins can be determined. Problems in the quantification of the saponins in leaf samples can be overcame with filters of different wavelengths.

Introduction

Most investigations on etioplasts also include experiments on structure and biochemistry of prolamellar bodies (PLBs) [1]. Recently new definitions of PLBs and of the adhering prothylakoids (PTs) as macromolecular systems with different compositions [1, 2] have been published. These investigations have shown that a separation of both systems is necessary before experiments with the respective complex are started. Often a determination of the content of PLB-tubules in an isolated subfraction of etioplasts is required. These tubules are mainly constructed of two saponins, Avenacosid A and Avenacosid B [3], which could not be found in considerable amounts in the prothylakoid membranes [4, 8] or in thylakoids of green plants [5]. So an evaluation of the saponin concentration in a given fraction is an indication of the content of the PLB tubules proper [4].

Up to now two different procedures to determine saponins after one purification step (TLC) are published [4, 5]. Despite they may be used routinuously, they are time – consuming [5] or require application of concentrated sulfuric acid [4]. Here we describe a quick and efficient method to determine a) the amount of tubules as well as their distribution in different fractions and to determine b) the concentration (in ng or µmol) of both Avenacosids by comparison with standard solutions of the isolated saponins.

Abbreviations: PLB, prolamellar body; PT, prothylakoid; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography.

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0341-0382/80/0500-0519 $ 01.00/0

Materials and Methods

Plants of *Avena sativa* were grown in total darkness at 23 °C and 85% rel. humidity. Harvesting of the 8–9 day old leaves and isolation of etioplasts were performed as described elsewhere [6]. To liberate PLBs from isolated etioplasts we used osmotic treatment of the plastids with an EDTA-buffer as is described in ref. [7]. Purification of PLBs was achieved by continuous sucrose gradient centrifugation using a Sorvall SS 90 Vertikal rotor (30 min, 35000 × *g*). Only the band containing PLBs was removed and washed twice with water (30 min, 35000 × *g*). The saponins of the PLB tubules were dissolved by extracting the pellets with 80% acetone. The resulting extract was used to isolate Avenacosid A and B according to the method described in ref. [3]. After purification the saponins were dried carefully and used to prepare the desired standard solutions.

TLC was performed on silica gel plates. The best results were obtained with HPTLC plates (Merck) in the solvent system $CHCl_3$ 70 MeOH 30 H_2O 5.5. The plates were sprayed with a phenol-sulfuric acid reagent [9] and developed for 10 min at 120 °C. To scan the chromatograms we used a Quick-Scan Junior densitometer (Helena Lab.) with exchangeable filters (485 nm and 645 nm) or white light. Scans were also run on a Vitatron-densitometer*. Peak areas were calculated by the integrators of the instruments.

To determine saponins in *Avena* leaves 1 g of leaves (upper half) was ground with sand in a mortar in the presence of 80% acetone, and the extraction repeated twice. A complete extraction of both Avenacosides could be obtained with $CHCl_3$/MeOH (1:2, v/v) in the last extraction step. For TLC the application of 10–50 µl from the combined

* I thank Prof. W. Rüdiger (Bot. Institut, Univers. München) for the possibility to use this instrument.

extracts was sufficient. To separate interfering spots in the leaf samples it is necessary to run the plate first in acetone/benzene/water (91:30:8).

Results and Discussion

Previous experiments have shown that investigations on isolated PLB- and PT-fractions cannot be carried out with preparations which are completely purified [1, 8]. This is the reason, why in isolated subetioplast fractions determinations of the PLB distribution between the fractions as well as calculations of recoveries are required. The method of choice is to measure the tubule-specific saponins. These measurements also may help to follow the construction or the disappearance of PLBs during different experimental conditions. Separations with TLC of acetone extracts from plastid fractions are very useful to analyze for saponin-respective tubule – concentration. By using HPTLC plates running time for each solvent is shortened to about 7 to 15 min, in addition sharpness and therefore separation of bands is improved. The saponins Avenacosid A and Avenacosid B are detected with a phenol reagent [9]. All sugar bands appear brownish on a white background and are in a good state for scanning. The color has a high absorption at 485 nm, because this kind of color development is similar to the reaction which also may be used to detect saponins and is measured in the photometer [4].

An instrument for scanning thin-layer plates enables one to determine the spot-intensity of the saponins quickly and accurately. A quantification of the values is possible, because both saponins were purified according to ref. [3] and could be applied in known amounts on the plates (Fig. 1). To investigate the content of tubules or saponins in an etioplast or subetioplast fraction aliquots of the standard solutions shown in Fig. 1 are applied to the same plate as the unknown samples. So all samples are run and scanned under identical conditions.

The calibration curves in Fig. 2 demonstrate that a sensitive evaluation of a saponin after densitometry is possible. The high sensitivity (lower limit about 0.5 nM or µg) allows a determination also in dilute extracts or in fractions with a low content of tubules. The curves showing nmol values were obtained after calculation of the molecular weights for the Avenacosids derived from the structural formulas given in ref. [3]. The following molec. weights were used: Avenacosid A: mw 1062.8, Avenacosid B: mw 1225.0.

To evaluate the concentration of saponins in leaves of etiolated *Avena sativa* needs a somewhat altered procedure. To get a complete recovery from the leaves an additional extraction with a few ml of $CHCl_3$/MeOH is required. Such an extract can also be used without difficulties for the quantitative determination of PChl(ide) or Chl(ide). Our experiments have shown that the addition of a few ml $CHCl_3$/MeOH do not shift the absorption maxima of the acetone extract at 626 nm and 663 nm nor alter the extinction coefficients. But leave samples contain unidentified compounds which appear yellow after spraying with the phenol reagent and which overlap the spots of both Avenacosids. If the

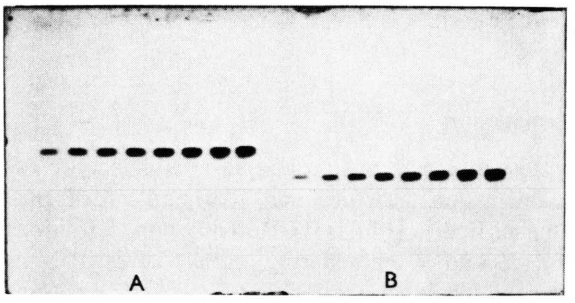

Fig. 1. Typical separation of the purified Avenacosid A and B on a HPTLC plate to obtain calibration curves (see Fig. 2). 1–8 µg of each saponin was applied.

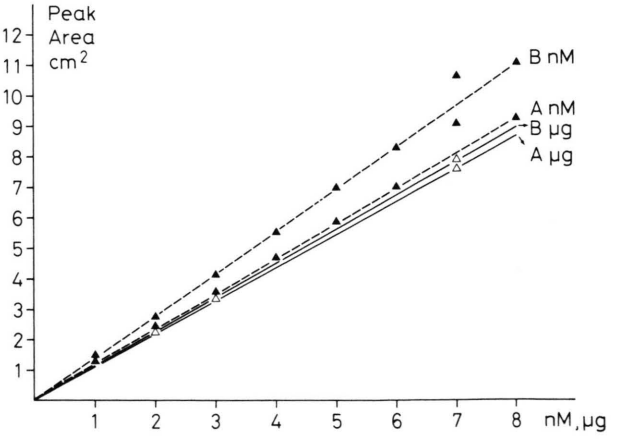

Fig. 2. Calibration curves of Avenacosid A and B after TLC-separation in $CHCl_3$ 70 MeOH 30 H_2O 5.5. The peak areas were measured with the integrator of the densitometer. The values for nanomoles were calculated as is described in the text.

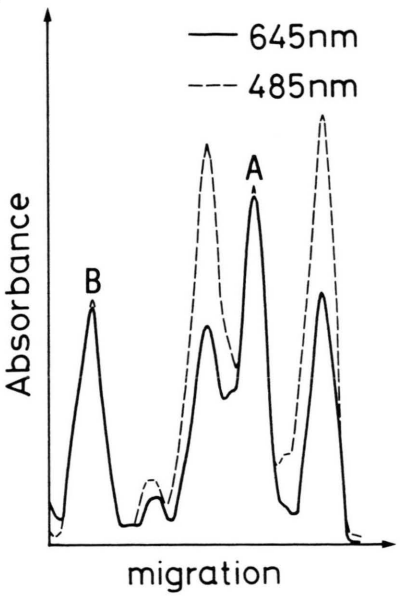

Fig. 3. Part of a scan of an Avena leaf extract. "A" and "B": Avenacosid A and B. The high peaks near by "A" correspond to unknown compounds in the extract. The determination of the peak areas of A and B are greatly facilitated by scanning at 645 nm.

plates are developed first in acetone/benzene/water, the separation allows scanning of the saponins. In most experiments a quantitative measurement of Avenacosid A and B needs a filter of 645 nm instead of a 485 nm filter as indicated above, because the yellow compounds interfere strongly at shorter wavelengths. Fig. 3 demonstrates the differences in the scans at 485 nm and at 645 nm in the interesting part of the separation. The area of the two peaks running before and after "A" is strongly reduced using 645 nm filter and enables a more correct determination of the concentration of "A".

Experiments on structure and function of the internal etioplast system should in general need also the determination of the amount of PLBs. Alterations of the content of tubules in etioplasts during development of etioplasts or while they are destroyed by light are also seen with measurements of the saponin concentration (*e. g.* [5]).

So the method described herein facilitates all these determinations. Isolation of etioplasts, PLBs and PTs and determinations of pigments and saponins in these fractions as well as in intact leaves are now carried out in one day only.

But if a densitometer is not accessible, the saponin determination as is described in ref. [4] allows also an accurate calculation.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft through SFB 74. The skillful technical assistance of Mrs. U. Männing is gratefully acknowledged.

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