Localization of the Tri- and Digalactosyl Diglyceride in the Thylakoid Membrane with Serological Methods

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(Z. Naturforsch. **31 c**, 589–593 [1976]; received July 19, 1976)

Trigalactosyl Diglyceride, Digalactosyl Diglyceride, Antibodies, Chloroplasts, Thylakoid Membrane

Trigalactosyl diglyceride was isolated from leaves of *Urtica dioica* and characterized by thin layer chromatography, infrared spectroscopy and by its fatty acid composition. An antiserum to the trigalactolipid was obtained by immunization of rabbits. By means of inhibition experiments with oligosaccharides and mono- and digalactosyl glycerol it was demonstrated that the antibodies are directed towards the α-galactosyl-(1 → 6)-α-galactosyl-(1 → 6)-β-galactosyl-(1 → 1)-glycerol configuration of the trigalactosyl diglyceride. Monogalactosyl diglyceride and sulfoquinovosyl diglyceride do not react with this antiserum. However, a cross reaction was observed with digalactosyl diglyceride. The presence of antibodies to tri- and digalactosyl diglyceride was demonstrated in antisera to different chloroplast preparations of *Antirrhinum majus* and *Spinacia oleracea*.

The antiserum to the trigalactolipid agglutinates stroma-freed chloroplasts. Membrane fragments obtained by the ultra sonication were precipitated. The antiserum is exhausted by trigalactosyl diglyceride but not by digalactosyl diglyceride or digalactosyl glycerol. The antiserum treated with digalactosyl glycerol and digalactosyl diglyceride also agglutinated stroma-freed chloroplasts. 1 g stroma-freed chloroplasts binds 0.17 g antibodies to trigalactolipid. Membrane fragments bind more antibodies to trigalactolipids than stroma-freed chloroplasts. From the agglutination tests it follows that the antigenic determinants of the trigalactolipid and the digalactolipid are localized in the outer surface as well as in the surface directed towards the inside of the thylakoid membrane.

In earlier publications we have reported on the localization of the chloroplast lipids monogalactosyl diglyceride [1], sulfoquinovosyl diglyceride [2–4], phosphatidyl glycerol [5], plastoquinone [6] and of the pigments chlorophyll a [7–9], lutein [6] and neoxanthin [10] in the thylakoid membrane. Antigenic determinants of these lipids are situated in the outer surface of the thylakoid membrane in a location which is accessible to antibodies. By immunization of rabbits we succeeded in obtaining an antiserum to the trigalactosyl diglyceride. The occurrence of the trigalactolipid as well as that of the mono- and digalactolipid has been demonstrated in chloroplasts of plants of different systematic positions [11–14]. According to Allen and co-workers [11] membrane lipids consist up to 3% of trigalactolipid. In the following we report on the isolation of the trigalactolipid, the preparation and characterization of the antiserum and on reactions of the antiserum with chloroplast preparations.

Materials and Methods

Isolation of the trigalactosyl diglyceride

From the mixture of the ether soluble lipids from *Urtica dioica* the phosphatides were precipitated with acetone. The acetone soluble fraction was loaded onto a silicic acid column. Elution was started with chloroform and continued with a chloroform/methanol gradient with increasing methanol concentrations (1–7%). With this gradient sequentially the pigments, neutral lipids, the galactolipids and the sulfolipids were eluted. Subsequently, the major amount of the trigalactolipid with traces of the digalactolipid and sulfolipid was eluted with methanol. This fraction was loaded onto a DEAE-column. The digalactolipid was eluted with a 9:1 (v/v) chloroform-methanol mixture and the trigalactolipid with a 6:4 chloroform-methanol mixture [12]. Subsequently, the trigalactolipid was rechromatographed on a silicic acid column as described above. The final purification was achieved on thin layer plates coated with silicia gel G with the solvent chloroform/methanol/acetic acid/water (85 : 15 : 10 : 3,5 v/v). After this step the trigalactolipid was extracted according to the method of Folch and co-workers [15] with several portions of chloroform/methanol (2 : 1 v/v) and then shaken with 0.45% saline. The purity of the preparation was tested by thin layer chromatography on silica gel G. The solvents used and the corresponding R_F-values are summarized in Table I. Staining agents used were: 5% phosphomolybdenic acid in ethanol, 80% sulfuric acid, 5% anilin diphenyl aminophosphate in ethanol and 0.13% anthrone in sulfuric acid.

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IR spectroscopy

The galactolipids were coated from benzene solutions onto silicium plates. After evaporation of the solvent the spectra were recorded in absorption mode with a Perkin-Elmer Infrarot-Gitterspektrometer-325.

Analysis of the trigalactolipid

The fatty acids were analysed by gas chromatography as methyl esters and the sugars as trimethyl silyl ethers according to earlier described methods [2]. The experiments were carried out with the gas chromatograph model type 5750 from Hewlett-Packard. The methyl esters were analysed on 10% ethylene glycol succinate and the trimethyl silyl ether of the sugars on 1.5% silicone SE 30 columns.

For the deacylation the trigalactolipid was treated with a 0.2 N methanolic KOH solution during 1 h at 40 °C [16] and subsequently passed through a Dowex 50-column (H-form). The identification of the hydrolysis product was made on silica gel G by thin layer chromatography. The chromatograms were run with butanol/acetic acid/water (50/40/10 v/v).

Preparation of the antiserum

10 mg trigalactolipid and 2 mg methylated bovine serum albumin were suspended in 1 ml physiological saline and emulgated with 1.5 ml Freund's adjuvant. This emulsion was injected subcutaneously into the hind leg of a rabbit. After 6 weaks a trigalactolipid-bovine serum albumin emulsion was injected in a two day rhythm i.v. into the animal. This emulsion contained 4 mg lipid and 1 mg methylated bovine serum albumin. After the 6th injection blood withdrawal was started. Further blood withdrawals followed in intervals of 5 days. Control sera were withdrawn from the animal before treatment. The serum was stored at − 16 °C.

Immune assay

The method for the passive heme agglutination test, the titer determination, the agglutination and adsorption tests, as well as the inhibition experiments with mono- and oligosaccharides have been described earlier [1, 3, 17]. The preparation of the ultrasonic sediment and the ultrasonic supernatant from stroma-freed chloroplasts has also been described earlier by Kannangara and van Wyk [18].

Test for IgM type antibodies

Stroma-freed chloroplasts were suspended in 0.06 M phosphate buffer pH 7.8 (2 mg/ml) and supplemented with the same amount of antiserum to trigalactolipid. After 12 h the chloroplasts were washed 14 times with physiological saline. The washed chloroplasts were suspended in phosphate buffer (1.5 mg/ml). This suspension with antibody sensitized chloroplasts was supplemented in parallel assays with equal volumes of an antiserum to rabbit-γ-globulines from either type IgG or IgM (Miles Laboratories Inc., Kankakee, Illinois). The test was made microscopically.

Results

1. Isolation and characterization of trigalactosyl diglyceride

The trigalactosyl diglyceride was isolated by chromatography on silicic acid and DEAE cellulose from the ether soluble lipids of *Urtica dioica* [2, 12, 19, 20]. The two-dimensional thin layer chromatography of this lipid on silica gel G yielded one spot. In the solvent butanol/acetic acid/water [21] the lipid exhibited the same R_F-value as the one prepared by Gent and Gigg [22]. The test was carried out by E. Heinz (University of Cologne). The R_F-values of the trigalactolipid run with different solvents are summarized in Table I. For comparison purpose also the R_F-values for mono- and digalactosyl diglyceride are listed.

Heinz [23] had reported that a lyso component of the digalactosyl diglyceride behaves similar to a trigalactolipid in the listed solvents. Therefore, the obtained galactolipid was deacylated with 0.2 N

Table I. R_F-Values of the mono-, di- and trigalactosyl diglyceride on silica gel G thin layers in various solvents.

Solvent	Volume ratio	R_F-Value Monogalactolipid	Digalactolipid	Trigalactolipid
Chloroform/methanol/water	65 : 25 : 4			0.52
Butanol/acetic acid/water	50 : 40 : 10			0.46
Chloroform/methanol/acetic acid ethylester/2% Ammonia	50 : 25 : 25 : 1.5	0.70	0.40	0.20
Chloroform/methanol/acetic acid/water	85 : 15 : 10 : 3.5	0.80	0.38	0.18
Chloroform/methanol/ammonia	75 : 21 : 4	0.61	0.12	0.06

KOH. The hydrolysis product showed in the solvent butanol/acetic acid/water just as the monogalactosyl glycerol and the digalactosyl glycerol one single spot. The R_F-values for the monogalactosyl glycerol were 0.3, for the digalactosyl glycerol 0.16 and for the trigalactosyl glycerol 0.08.

The IR-spectra of the tri-, di- and monogalactolipid from *Urtica dioica* are shown in Fig. 1. The

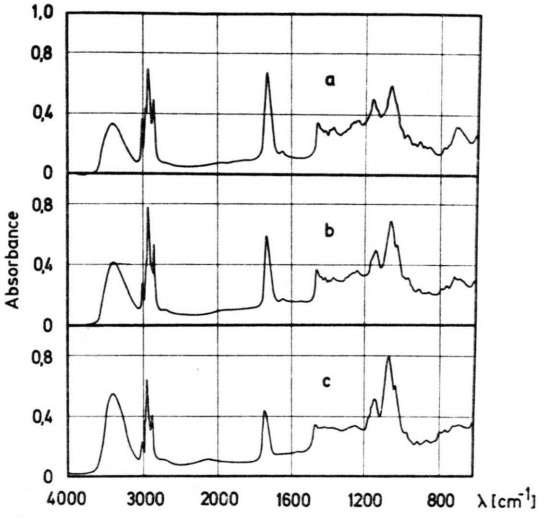

Fig. 1. IR-Spectra of the galactolipids from *Urtica dioica*, a) monogalactosyl diglyceride; b) digalactosyl diglyceride; c) trigalactosyl diglyceride.

comparison shows, that the extinction ratio of the hydroxyl band at 3400 cm^{-1} as well as that of the alkohol band at 1070 cm^{-1} in comparison to the ester carbonyl band at 1740 cm^{-1} is largest for the trigalactolipid [12, 24]. From this it follows that in this lipid the sugar portion is larger than in the di- and monogalactolipid.

By gas chromatography the sugar component was identified as galactose. The gas chromatographic fatty acid analysis on ethylene glycol succinate *via* the methyl esters showed that the trigalactolipid is rich in polyenic acids (Table II). The major component is as with the mono- and digalactolipid linolenic acid. However, the trigalactolipid differs from the other two galactolipids in its larger palmitic acid content. Whereas the monogalactolipid only contains fatty acids with 18 carbon atoms, these acids make up only for 90% in the digalactolipid and only for 83% of the fatty acid content in the trigalactolipid. The digalactolipid stands in its fatty acid composition between the mono- and trigalactolipid.

Table II. Fatty acid composition of the galactolipids from *Urtica dioica* in per cent total fatty acids.

Number of carbon atoms and number of double bonds	Monogalactosyl diglyceride	Digalactosyl diglyceride	Trigalactosyl diglyceride
$C_{14:0}$	—	—	0.4
$C_{16:0}$	1.4	9.4	16.5
$C_{18:0}$	3.4	2.5	4.3
$C_{18:1}$	0.5	0.5	1.9
$C_{18:2}$	4.5	4.0	4.7
$C_{18:3}$	90.2	83.6	72.2

According to Allen and co-workers [11] and Webster and Chang [25] the trigalactolipid from spinach leaves has a similar fatty acid composition. In a trigalactolipid from seeds of *Glycine hispida*, which was isolated for comparison purpose, linoleic acid with 50% of the total fatty acids prevails just as with mono- and digalactosyl diglyceride from other plant depot organs [20].

The average molecular weight of the trigalactolipid from *Urtica* leaves was calculated to be 1180.

2. *Presence of antibodies to lipids and proteins in antisera to chloroplasts*

Complex antisera are obtained if rabbits are immunized with stroma-freed chloroplasts and chloroplast preparations from *Antirrhinum majus* and *Spinacia oleracea*. These antisera contain andibodies to proteins [18, 26–34], lipid [1–5] and glykolipopeptides [35] of the thylakoid membrane. The presence of antibodies to proteins was demonstrated by precipitation reaction, immune electrophoresis and double diffusion [36]. The demonstration of the presence of antibodies to monogalactosyl diglyceride, sulfoquinovosyl diglyceride and phosphatidyl glycerol as well as to chlorophyll a was achieved by means of the passive heme agglutination test [1, 3, 5, 7]. With the same method we also tested for antibodies to the described trigalactosyl diglyceride. The formation of antibodies took place during the first booster immunization with stroma-freed chloroplasts. The titer after the third booster immunization with stroma-freed chloroplasts was 1 : 250 and that in the immunization with chloroplast fragments of *Antirrhinum majus* and *Spinacia oleracea* 1 : 500.

3. *Specificity of the antiserum to trigalactosyl diglyceride*

The antiserum to trigalactosyl diglyceride exhibited in the passive heme agglutination test a titer

of 1:128. The antiserum reacts, not only with the trigalactolipid from *Urtica dioica* used for immunization, but also with the trigalactolipid from blue green algae and from *Glycine hispida* although these lipids differ with respect to their fatty acid composition. A cross reaction was observed with the digalactolipid but did not occur with the monogalactolipid. The antiserum did also not react with the sulfolipid. With a partially synthesized digalactosyl dioleyl glycerol the passive heme agglutination test was also positive. Hydration of the unsaturated fatty acids had no influence on the reaction. After oxidation of the trigalactolipid and the digalactolipid no agglutinations were observed anymore.

Inhibition experiments with saccharides have led to the result, that the oligosaccharides stachyose and raffinose inhibit the passive heme agglutination test at concentrations of 0.02 M/ml up to 50%. In these oligosaccharides the terminal galactose is bound in the 1,6 position in the α-glycosidic form. In contrast to this, lactose which contains the galactose in β-glycosidic form had no influence. From the monosaccharides only galactose in the above mentioned concentration gave an inhibitory effect of 20%. Mannose, glucose and arabinose even in 50 fold concentration were of no effect. The highest inhibition was achieved with digalactosyl glycerol which was derived from the digalactolipid by mild alkaline hydrolysis. At the concentration of 0.02 M/ml the inhibition was 80%. Monogalactosyl glycerol, which was also obtained from the monogalactolipid by hydrolysis had no influence on the reaction. An exhaustion of the antiserum was only obtained with trigalactosyl diglyceride and occurred already at a concentration of 0.04 μM/ml. On the other hand the digalactolipid inhibited only up to 80% at a concentration of 0.02 M/ml.

From these inhibition experiments it follows that the antibodies are directed towards the α-galactosyl-(1→6)-α-galactosyl-(1→6)-β-galactosyl-(1→1)-glycerol-configuration in the trigalactosyl diglyceride. The fatty acids do not affect the specificity.

4. *Reactions of the antiserum with chloroplast preparations*

The antiserum to trigalactolipid agglutinates stroma-freed chloroplasts from *Antirrhinum* with a titer of 1:128 (Table III). Membrane fragments, obtained by ultrasonication, react with the antiserum with a considerably lower titer than untreated chloroplasts (Table III). These agglutination reactions were fully avoided by the addition of a trigalactolipid emulsion. With digalactosyl diglyceride, however, the antiserum was not fully exhausted. Inhibition experiments with digalactosyl glycerol had the same result as in the passive heme agglutination test. If chloroplasts were treated with sodium periodate no agglutination was observed. From these experiments it can be concluded that tri- and digalactolipid molecules are located in the outer surface as well as in the surface directed towards the inside of the thylakoid membrane.

Table III. Reactions of the antiserum to trigalactosyl diglyceride with stroma-freed chloroplasts from *Antirrhinum majus* as well as with ultrasonic sediment and ultrasonic supernatant.

Chloroplast preparations	Reaction	Agglutination and precipitation	Amount of antigen in mg which exhausts 1 ml of antiserum
Stroma-freed chloroplasts	agglutination	1 : 128	12
Ultrasonic sediment	agglutination	1 : 16	7
Ultrasonic supernatant	precipitation	1 : 4	0.5

For the agglutination reaction and the determination of the titer chloroplasts were suspended in 0.06 M phosphate buffer and adjusted to a concentration of 1.5 mg/ml.

According to an earlier described method [17] the maximal binding of antibodies to trigalactolipid onto stroma-freed chloroplasts was determined. The result was that 1 g chloroplast was able to bind 0.17 g antibodies. When comparing the values for maximally bound antibodies with other components of the thylakoid membrane it must be borne in mind that in the present case the antibodies are of the γ-globuline type IgM and not of the IgG type. This was demonstrated with the antiglobuline test according to Coombs *et al.* [37] and according to Uhlenbruck [38]. It should be noted that antibodies to the monogalactolipid also belonged to the immune globuline class IgM. On the other hand the antibodies to the anionic sulfolipid and phosphatidyl glycerol as well as those to the xanthophylls lutein and neoxanthin are of the IgG type.

The author would like to thank Prof. Dr. W. Menke for helpful discussions and Prof. Dr. G. Uhlenbruck, Abt. für Immunobiologie der Medizini-

schen Universitätsklinik, Köln-Lindenthal, for useful suggestions. Digalactosyl glycerol and monogalactosyl glycerol was a gift from Prof. Dr. E. Heinz, Botanisches Institut der Universität, Köln, and Dr. Lekim from the Natterman AG, Köln, kindly provided the lipid extract from seeds of *Glycine hispida*. The technical assistance of Mr. W. Schmalenbach is also acknowledged.

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