Structures and molecular dynamics of plant waxes

II. Cuticular waxes from leaves of *Fagus sylvatica* L. and *Hordeum vulgare* L.

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Abstract. Waxes from the leaves of *Fagus sylvatica* L. (European beech tree) and *Hordeum vulgare* L. (barley) have been investigated using NMR, DSC, X-ray diffraction and gas chromatographic methods. The wax from *Fagus sylvatica*, consisting mainly of n-alkanals, n-alkanes and 1-alkanols, has chain-lengths ranging from 20 to 52 carbon atoms with an average chain-length of 30.5 carbon atoms. The X-ray results show that the wax is to a large extent (~70%) amorphous. The wax from the leaves of *Hordeum vulgare* L., consisting mainly of n-alkanols, has chain-lengths ranging from 20 to 50 carbon atoms with an average chain-length of 27.4 carbon atoms. The wax is ~52% crystalline. It seems that the structure of this wax differs from those of other plant waxes that have been investigated in that the longer chains bridge the amorphous zone between two adjacent layers of crystalline material, thus linking the two layers. This linking affects the melting point of the wax noticeably. The activation energies for the different molecular motions in these waxes have been extracted from the NMR spin-lattice relaxation time measurement.

Key words: Cuticular wax – Chain-length distribution – Molecular motions – Crystallinity – Phase transitions

1. Introduction

The plant cuticle is a very thin skin that covers the leaves, fruits, stems and flowers of higher plants and serves as a protective barrier to the environment, controlling excessive water and nutrient losses. The matrix of this extracellular lipophilic membrane consists of the biopolymer cutin which is essentially an amorphous polyester of hydroxyalkanoic acids (Kolattukudy 1980; Holloway 1982). Cuticular waxes embedded within this matrix and deposited on its outer surface, consist of a mixture of long-chain aliphatic components (Baker 1982).

These waxes represent the main barrier to the diffusive transport of water and solutes across the plant cuticle. Permeabilities increased by two to four orders of magnitude when the waxes were removed by organic solvents (Schönherr 1982; Schönherr and Riederer 1989). There have been numerous attempts to correlate the transport properties of plant cuticles with the chemical composition of their waxes. Recently Riederer and Schneider (1990) suggested that crystalline arrangements of the aliphatic chains of the wax molecules may form the transport limiting barrier of the cuticle. Thus the physical structure of the wax should be a more important determinant of transport across the cuticle than the chemical composition thereof. Consequently it was recognised that the phenomenological description of the transport properties of plant cuticles should be supplemented by the quantitative analysis of the cuticular waxes and the investigation of the physical principles determining these properties.

The composition of the cuticular wax of leaves of *Citrus aurantium* L. (Riederer and Schneider 1990) and various physical properties of plant cuticles such as degree of order and orientation of intracuticular wax deposits, thermal properties of the cuticular matrix membrane and waxes (Kreger 1958; Sitte and Renner 1963; Eckl and Gruler 1980; Schreiber and Schönherr 1990), have been investigated. More recently cuticles and wax from the leaves of *Citrus aurantium* L. were investigated by Reynhardt and Riederer (1991) using NMR, DSC, X-ray diffraction and gas chromatographic methods. This paper and the present one are Parts I and II, respectively, of a series of papers on the structures and molecular dynamics of plant waxes. Citrus wax, consisting mainly of 1-alkanols and n-alkyl esters, has chain-lengths ranging from 25 to 53 carbon atoms with an average chain-length of 34 carbon atoms. The wax is to a large extent (~80%) amorphous and the crystalline fraction seems to consist...
mainly of the n-alkyl esters with an average chain-length of 43.5 carbon atoms. The activation energies for the different molecular motions in the wax were extracted from the NMR spin-lattice relaxation time measurements. It was concluded that the presence of wax molecules does not influence the molecular dynamics of the matrix membranes noticeably.

In this paper the composition, structures and molecular dynamics of cuticular waxes extracted from leaves of the European beech tree (*Fagus sylvatica* L.) and barley (*Hordeum vulgare* L.) are investigated.

2. Experimental details

2.1. Sample preparation

Leaves were harvested on 1989/05/20 from a 60 to 80 year old *Fagus sylvatica* L. tree at Schachtenau at an elevation of about 800 m above sea level in the Bayerischer Wald National Park, E. Bavaria, Germany. At that time (24 days after budbreak) the leaves had attained their final size.

Plants of *Hordeum vulgare* L. (cultivar Andrea) were cultivated in growth chambers under controlled conditions. Fully developed leaves were harvested when the plants had more than three leaves unfolded but did not yet start tillering.

Cuticular waxes were extracted from whole leaves as described previously (Riederer and Schneider 1990) by immersing the leaf blades in chloroform (> 99%) for 30 min at 303 K. Average extraction efficiencies were about 90% of the total wax present in fresh leaves.

Samples used in NMR experiments were sealed off in glass ampoules after having been evacuated for several hours.

The mass of the DSC, X-ray diffraction and NMR samples was ~3 mg, ~100 mg and ~30 mg, respectively.

2.2. Analysis of cuticular wax components

For the qualitative determination of the cuticular wax components the wax extracts were separated into compound classes by thin-layer chromatography (TLC) on precoated Kieselgel 60 plates with cellulose acetate concentrating zones. TLC was performed under unsaturated sandwich conditions without control of temperature and relative humidity in the chamber. Separation was achieved by consecutive developments with cyclohexane and 1,1,1-trichloroethane.

The components of the waxes were indentified by capillary gas chromatography-mass spectrometry, performed under chromatography conditions as described below, with the exception that helium was used as carrier gas instead of hydrogen. A Hewlett-Packard Series II gas chromatograph with a 5971A mass selective detector was used. Mass spectra were taken in the electron impact mode and identified by comparison with published spectra or with spectra obtained from reference substances.

For qualitative analyses the cuticular waxes extracted from leaves were used without prior separation by liquid chromatographic methods. For derivatisation the solvent of the wax solutions was evaporated in a gentle stream of nitrogen gas and the samples treated with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine in a solution of dry pyridine and methanol (7:3 v/v) for 3 hours at 333 K. After cooling to room temperature, 10 μl of 2,2-dimethoxypropane was added and the samples were heated again for 2 min at 333 K. After cooling the solvent was evaporated in a stream of nitrogen gas and the samples treated with N,N-bis-trimethylsilyl trifluoroacetamide in dry pyridine for 30 min at 343 K. Before injection the reaction mixture was diluted as appropriate with chloroform.

Temperature programmed capillary gas chromatography was carried out on a gas chromatograph equipped with a flame ionization detector on an on-column injector. Fused silica WCOT columns (25 mm × 0.32 mm inner diameter covered with 0.13 μm CP-Sil 5 CB) were used. The temperature program was as follows: injection at 323 K, 2 min at 323 K, 40 K min⁻¹ up to 473 K, 2 min at 473 K, 3 K min⁻¹ up to 573 K, 40 min at 573 K, 3 K min⁻¹ up to 593 K. The inlet pressure of the hydrogen carrier gas was adjusted to 40 kPa at the beginning of each run and raised to 150 kPa 42 min after the injection. Where appropriate peak areas were corrected for differential response.

2.3. DSC

DSC thermograms of the waxes and a number of n-alkanes, n-hexacosane (n-C_{26}), n-octacosane (n-C_{28}) and n-letratetracontane (n-C_{44}), were obtained using a Stanton Redcroft 700 instrument. With a view to eliminating possible effects of moisture on the thermograms, they were obtained in a dry nitrogen atmosphere. Heating rates were 10 K min⁻¹.

2.4. Powder X-ray diffraction

The diffraction patterns for the waxes and n-C_{28} were obtained at room temperature with Cu Kα radiation. A Seifert MZIV diffractometer with a proportional counter was used. 2θ was varied at a rate of 0.25° min⁻¹.

2.5. NMR

The proton spin-lattice relaxation times in the laboratory frame, T₁, were measured at 200 MHz on a Bruker CXP200 pulse spectrometer by applying a train of sixteen close-spaced 90° pulses, followed at a variable interval τ by a single 90° measuring pulse. Proton T₁ values were obtained from the slopes of semi-log plots of (M - M₀)/M₀ versus τ, where M₀ is the equilibrium signal intensity and M the signal intensity a time τ after the saturating pulse sequence.

Proton spin-lattice relaxation time measurements in the rotating frame, T₁ο, were also made on the Bruker