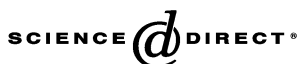


Available online at www.sciencedirect.com

Biochimica et Biophysica Acta 1674 (2004) 1–3



Rapid report

Plant biopolyester cutin: a tough way to its chemical synthesis

José J. Benítez^a, Rafael García-Segura^b, Antonio Heredia^{b,*}^a*Instituto de Ciencia de Materiales de Sevilla, CSIC, Universidad de Sevilla, Spain*^b*Grupo de Caracterización y Síntesis de Biopolímeros Vegetales, Departamento de Bioquímica, Biología Molecular y Química Orgánica, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, E-29071 Málaga, Spain*

Received 10 February 2004; received in revised form 25 May 2004; accepted 14 June 2004

Available online 4 July 2004

Abstract

The chemical synthesis of an aliphatic biopolyester identical to the natural cutin which constitutes the major component of the cuticle of fruits and leaves of higher plants is for the first time achieved and reported. Potential applications of this new material is of great interest because its physical properties, non-toxicity, biodegradability, and availability of raw material.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Polyester; Plant cutin; Chemical synthesis

1. Introduction

Cutin is a support biopolyester involved in waterproofing the leaves and fruits of higher plants. Cutin is the main component (between 40% and 85%, w/w) of the plant cuticle, the continuous and lipidic extracellular membrane that covers the aerial parts of leaves, fruits and non-lignified stems of plants. Plant cuticular material occurs in large amounts in both natural and agricultural plant communities: between 180 and 1500 kg per hectare. Considering the average weight of an isolated cuticle (around 600 $\mu\text{g cm}^{-2}$), cutin can be considered the major lipid plant polymer.

From a chemical point of view, cutin is defined as a polymeric network of polyhydroxylated C_{16} and C_{18} fatty acids cross-linked by ester bonds [1]. A suite of physical, chemical and morphological properties gives the plant cutin the characteristics of a unique and complex biopolymer. These properties have been recently summarized and discussed in a comprehensive review [1]. Cutin is an amorphous and insoluble polymer; it presents a very low water sorption and permeability [2], high specific heat and shows glass transition events in the range of physiological or ambient temperatures [3]. Moreover, cutin has interesting rheological properties: it can be considered as a viscoelastic

polymer network [4]. We are now beginning to understand the physics, chemistry and biology of plant cutin. Since 400 millions years ago, this biopolymer conducted the vascular plants to establish on dry land, minimizing water loss and supplying them a powerful, resistant and flexible chemical barrier just at the interface between the epidermal plant cells and the atmosphere.

The above mentioned characteristics places this unique polyester in a different position in comparison to other polymers that currently attract industrial and biotechnological attention as polyhydroxyalkanoates (PHA) or other thermoplastic polyesters obtained after chemical or enzymatic polymerizations. Also, biodegradable polymers have gained considerable significance in medical and industrial applications. Here, we report some preliminary results on the first chemical synthesis of a polyester with the same molecular structure and characteristics that the common cutin isolated from cuticles of tomato fruits.

2. Materials and methods

2.1. Monomer isolation and analysis

Cuticles were isolated from astomatous tomato fruits of greenhouse-grown *Lycopersicon esculentum* Mill. as described in Ref. [4]. Cutin samples were obtained after hydrolysis of dewaxed cuticles in a 6 M HCl solution for

* Corresponding author. Tel.: +34-952-131-940; fax: +34-952-132-000.
E-mail address: heredia@uma.es (A. Heredia).

12 h at 105 °C to remove polar hydrolyzable components and then depolymerized in a 3% (w/v) sodium methoxide solution for 18 h at 100 °C. After extraction of the monomers of tomato fruit cutin in an organic phase (diethyl ether), the solvent was evaporated to quantify and identify the cutin monomers by gas chromatography-mass spectrometry analysis after silylation with *N*-*O*-bistrimethylsilylacetamide, using a Hewlett-Packard 5890 GC-MS combination with a cross-linked methyl silicone capillary column.

2.2. Chemical synthesis

The polycondensation procedure between monomers of tomato fruit cutin is similar to that described by Saam [5]. In a typical run, around 350 mg of solid monomer mixture, 125 mg of the surfactant-type catalyst dodecylbenzenesulfonic acid (DBSA, Aldrich, Germany) and 2.5 ml of toluene were introduced into a 10 ml glass vial equipped with a magnetic stirrer. The mixture was briefly heated to about 75–80 °C with stirring in order to dissolve the monomers and catalyst. After cooling, the open glass vial is heated at 60–65 °C, the mixture was continuously stirred and a gentle flow of nitrogen was passed over the surface of the solution. Typically, there was some minor loss of organic solvent, the addition of a few microliters of toluene to maintain the volume of reaction constant being necessary. In these conditions, after 2 h of reaction, the polymer precipitates. The toluene was removed and the solid polymer was washed several times with toluene and methanol in order to remove the excess of the acid catalyst. The product, a pallid yellow film, was dried in vacuum at room temperature.

2.3. Fourier transform infrared (FT-IR) spectroscopy

KBr pellets were prepared using about 1.5 mg of solid product. Infrared spectra were recorded in a Perkin-Elmer 1760 FT-IR spectrometer.

2.4. Solid-state ¹³C-NMR spectroscopy

¹³C-Cross polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectra were recorded on a Bruker 300 MHz NMR operating at 75.5 MHz using an air-bearing probe. Experiments were conducted on about 30–40 mg samples of powdered samples using liquid nitrogen in a 5 mm MAS probe at room temperature.

2.5. Atomic force microscopy (AFM)

AFM images were obtained with a Topometrix TMX2000 microscope operating either in contact constant force mode with both amplitude and phase detection. A large-scale scanner (maximum *X*–*Y* range of 130 × 130 and 13 μm in *Z*) was used to analyze the overall texture and the homogeneity of the surface of the corresponding samples. When higher lateral and vertical resolutions were needed, another

scanner with maximum *X*–*Y*–*Z* ranges of 2.3 × 2.3 and 0.8 μm was used. In any case, the same Si₃N₄ lever (contact NanoProbe™, Digital Instruments, Santa Clara, CA) with 0.58 N/m nominal constant force was employed. In non-contact mode, a stiff Si₃N₄ lever (NT-MDT Ultrasharp NSCS12) oscillated at its resonance frequency (approx. 149 KHz) is used.

Calibration in the *X*–*Y*–*Z* directions was done with commercial calibration gratings provided by formerly NT-MDT, Moscow. Samples were attached to a glass slide using a double side adhesive tape and analyzed at room atmosphere, typically 20–25 °C and 45–50 % relative humidity.

3. Results and discussion

Two main characteristics present our experimental approach: (1) the mixture of monomers has been isolated

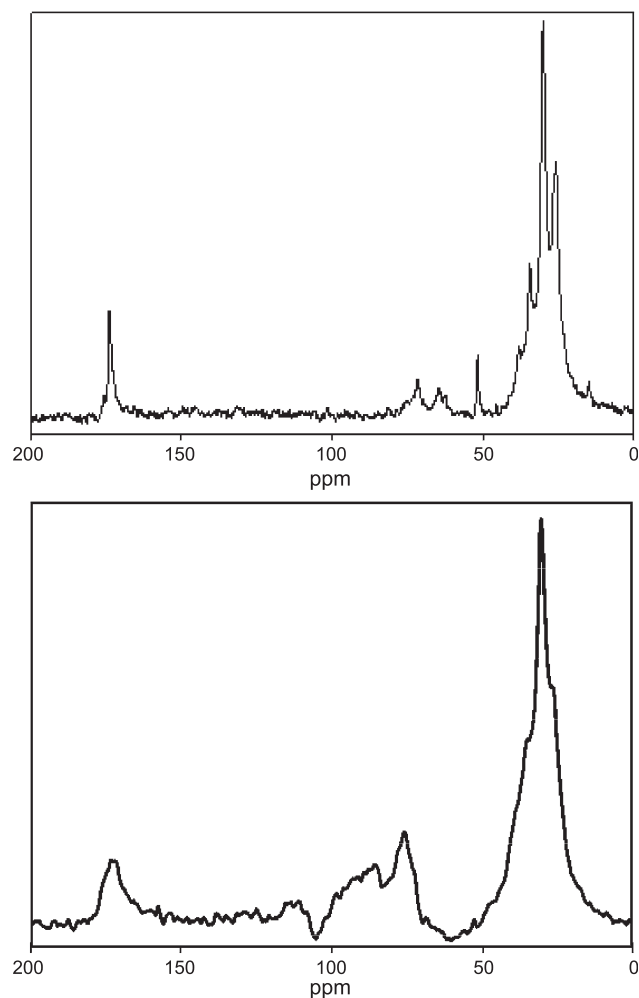


Fig. 1. ¹³C CPMAS NMR spectra of the cutin-like polymer synthesized (upper spectrum) and cutin extracted and purified from epidermis of 19-day-old tomato fruits (lower spectrum). The main chemical shift assignments are as follows: $(\underline{\text{C}}\text{H}_2)_n$, 29 ppm; $\text{CH}_2\underline{\text{C}}\text{H}_2\text{OCOR}$, 64 ppm; $\underline{\text{C}}\text{HOCOR}$ and $\underline{\text{C}}\text{HOH}$, 71.6 ppm; carbonyl (ester linkages), 173 ppm.

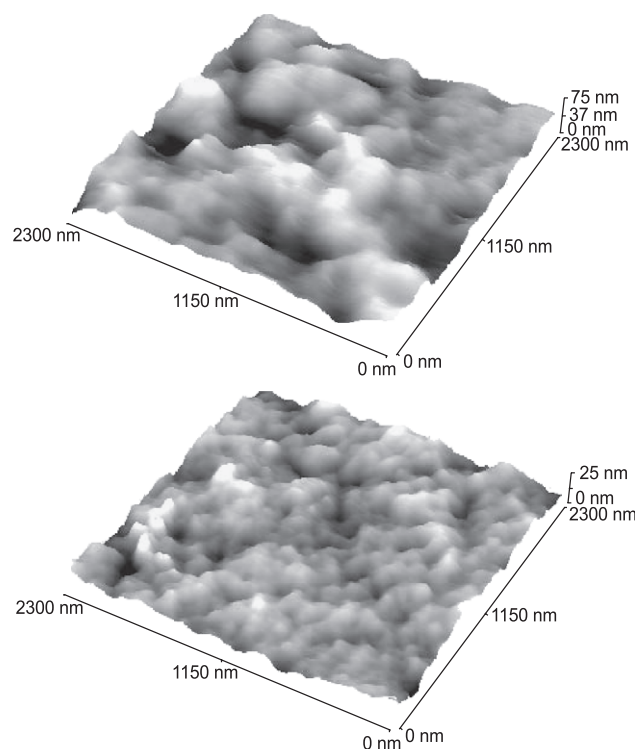


Fig. 2. AFM images ($2.3 \times 2.3 \mu\text{m}$) obtained in contact mode in air of synthetic cutin (upper image) and natural cutin extracted from the epidermis of 19-day-old tomato fruits (lower image).

from a cheap source: the peel and their corresponding isolated cuticles of commercial tomato fruits; cutin from these cuticles mainly consist in a mixture of 9(10),16-dihydroxyhexadecanoic acid (85%, w/w), 16-hydroxyhexadecanoic acid (7.5%, w/w); (2) experimental reaction conditions are soft in order to avoid unfavorable side reactions: the monomers condense in toluene at 60–65 °C when the solution interface with an acidic hydrophilic phase, the surfactant-type catalyst DBSA. In this heterogeneous media, a dehydration esterification occurs [5]. Free energy released in the transfer of water from the hydrophobic phase to the hydrophilic phase, can be considered as the driving force for the esterification process. In addition, polymer yield is higher when the by-produced water is partly removed by passing a slow stream of nitrogen over the surface of the polymerizing monomer solutions [5].

The product, with a polymerized film aspect, was insoluble in a high number of organic solvents tested. Only in the presence of hot basic methanol (KOH/MeOH, 2% w/w) was the polymer depolymerized. X-ray diffraction analysis (data not shown) indicated the amorphous nature of the polymer. FT-IR spectrum, with an intense and symmetrical C=O stretch at 1736 cm^{-1} , confirmed the formation of ester bonds. Moreover, other infrared bands of the FT-IR spectrum were identical to that found in cutin samples isolated from fruit and leaf cuticles [6]. Solid-state ^{13}C -NMR spectrum of the synthesized polymer also presented the typical

resonances of isolated and purified plant cutin samples [7]. This similarity is shown in Fig. 1 after comparison of both types of products.

Besides the chemical characterization, and from a physical point of view, the similarity between synthetic and natural cutin is further extended to their surface texture, as revealed by AFM (Fig. 2). Surface texture is an important parameter that is directly related with important physical properties of cutin such as its rheological and mechanical behavior. Both surfaces are quite flat and show a globular texture (Fig. 2). Though grain size and overall roughness is higher for the synthetic cutin, values are similar to those obtained for cutin samples extracted from tomato fruits.

It is noticeable that the cutin-like polymer investigated here was only formed under the above mentioned conditions. Attempts made using acid resins, other acid catalysts and commercial lipases only produced soluble and short linear oligomers, mainly formed by heat-tail condensations. Finally, we would like to stress that the cutin-like polymer synthesized is potentially modifiable in order to realize future designs that take into account criteria such as degradation mechanisms, low density of cross-linking, and the incorporation of other non-toxic monomers (e.g. glycerol) in the structure. Other attractive polymer characteristics such as biodegradability and useful mechanical and thermal properties are inherent in its structure. At the same time, the mechanism of synthesis reported here gives an initial and alternative basis for the explanation of one of the unresolved paradigms of lipid plant biochemistry [1]: how this unique barrier biopolymer that constitutes the cutin is formed. The old hypothesis concerning the co-existence of both, the enzymatic and chemical ways, to explain the synthesis *in vivo* of the natural cutin must be reconsidered and investigated.

References

- [1] A. Heredia, Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer, *Biochim. Biophys. Acta* 1620 (2003) 1–7.
- [2] M. Riederer, L. Schreiber, Protecting against water loss: analysis of the barrier properties of plant cuticles, *J. Exp. Bot.* 52 (2001) 2023–2032.
- [3] C.G. Casado, A. Heredia, Specific heat determination of plant barrier lipophilic components: biological implications, *Biochim. Biophys. Acta* 1511 (2001) 291–296.
- [4] P.D. Petracek, M.J. Bukovac, Rheological properties of enzymatically isolated tomato fruit cuticle, *Plant Physiol.* 109 (1995) 675–679.
- [5] J.C. Saam, Low temperature polycondensation of carboxylic acids and carbinols in heterogeneous media, *J. Polym. Sci., A* 361 (1998) 341–346.
- [6] F. Villena, E. Domínguez, D. Stewart, A. Heredia, Characterization and biosynthesis of non-degradable polymers in plant cuticles, *Planta* 208 (1999) 181–187.
- [7] X. Fang, F. Qiu, B. Yan, H. Wang, A.J. Mort, R.E. Stark, NMR studies of molecular structure in fruit cuticle polyesters, *Phytochemistry* 57 (2001) 1035–1042.