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Molecular characterization of the plant biopolyester cutin by AFM and spectroscopic techniques

José J. Benítez,[a] Antonio J. Matas,[b] and Antonio Heredia[b,*]

[a] *Instituto de Ciencia de Materiales, CSIC-Universidad de Sevilla, E-41092 Seville, Spain*
[b] *Grupo de Caracterización y Síntesis de Biopolímeros Vegetales, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, E-29071 Málaga, Spain*

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Abstract

Atomic force microscopy, FT-IR spectroscopy, and solid-state nuclear magnetic resonance have been used to improve our current knowledge on the molecular characteristics of the biopolyester cutin, the main component of the plant cuticle. After comparison of samples of cutin isolated from young and mature tomato fruit cuticles has been possible to establish different degrees of cross-linking in the biopolymer and that the polymer is mainly formed after esterification of secondary hydroxyl groups of the monomers that form this type of cutin. Atomic force microscopy gave useful structural information on the molecular topography of the outer surface of the isolated samples. The texture of these samples is a consequence of the cross-linking degree or chemical status of the polymer. Thus, the more dense and cross-linked cutin from ripe or mature tomato fruit is characterized by a flatter and more globular texture in addition to the development of elongated and orientated superstructures.

Keywords: Biopolymer; Cutin; Molecular architecture; AFM topography

1. Introduction

Aerial parts of higher plants are covered by a continuous extra-cellular layer, the cuticle. The main function ascribed to the cuticle is to minimize water loss. Besides, it limits the loss of substances from plant internal tissues and also protects the plant against physical, chemical, and biological impacts (Holloway, 1982).

Cuticles of higher plants are chemically heterogeneous in nature, basically consisting of a wax fraction, soluble in common organic solvents, and an insoluble cuticular matrix, that forms the framework of the cuticle. This cuticular matrix is mainly formed by the biopolymer cutin, a high-molecular weight polyester composed of various inter-esterified C_{16} and C_{18} hydroxyalkanoic acids (Walton, 1990). The ester bonds in cutin can be cleaved by alkaline hydrolysis to yield the corresponding hydroxy fatty acids. Further compositional analyses have established a few classes of monomers which are present in cutin. Depolymerization products of many plant cutins are composed almost exclusively of derivatives of the C_{16} family of monomer acids, in which 10,16- and/or 9,16-dihydroxyhexadecanoic acids are the major compounds (Kolattukudy, 1996; Walton, 1990). Relatively few cutins contain significant amounts of C_{18} monomers (Kolattukudy, 1996).

The understanding of the types of covalent linkages in plant cutins has been based on the chemical reactivity of the biopolymer. On the other hand, the intermolecular cross-linking between cutin monomers has been derived from analysis of the abundance of free primary and free secondary mid-chain hydroxyl functional groups, as well as unesterified carboxyl moieties. Studies involving these approaches demonstrated that, in most cases, about half the mid-chain hydroxyl groups in the biopolymer are involved in ester linkages (Deas and Holloway, 1977). Structural studies on cutin have been previously reported by our research group being mainly focused on Fourier-transform infrared (FT-IR)

* Corresponding author. Fax: +34-95-213-2000.
E-mail address: heredia@uma.es (A. Heredia).

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spectroscopical analysis (Luque et al., 1995; Ramírez et al., 1992) and X-ray diffraction analysis (Luque et al., 1995). They suggested an amorphous structure with basal spacing around 0.45 nm as repeated unit in the macromolecular structure of cutin. Additionally, solid-state ^{13}C nuclear magnetic resonance (NMR) studies of the polyester provided structural information of the intact biopolymer, in which distinct polymer domains have been identified (Fang et al., 2001; Zlotnik-Mazori and Stark, 1988). On the other hand, such studies have led to the identification and quantification of the principal chemical functionalities of the cutin polymer. Structural and motional characteristics of the major carbon types were also obtained. A more detailed explanation of the physical characteristics and properties of plant cutin can be found in a recent review (Heredia, 2003).

The rheology of the plant cuticle and cutin is of particular interest. It is known that the diffusion and sorption across polymers is influenced by the mechanical properties of the polymer itself. Some factors that affect these properties are the polymer density, the presence of fillers and plasticizers in the polymer matrix, and the humidity and temperature. There is only one previous study concerning the nanomechanical behavior of the plant cutin by atomic force microscopy (Round et al., 2000). This technique was used to evaluate the surface elastic modulus of tomato fruit cutin in response to changes in humidity.

In the present work, a study on the molecular architecture of plant cutin in relation to its chemical composition has been made. Tomato fruit cutin isolated from fruits at different growth stages have been used to investigate the influence of the cross-linking degree and the subsequent macromolecular arrangement in the cutin polyester. For this purpose, atomic force microscopy (AFM), in combination with IR and NMR spectroscopies, has been used. These data draw a more complete picture of the cutin ultrastructure and also gives a molecular basis to understand the physical properties of this unique biopolymer.

2. Materials and methods

2.1. Cuticle an cutin isolation and analysis

Cuticles were prepared from astomatous tomato fruits of greenhouse-grown *Lycopersicon esculentum* Mill. Fruits were collected at two different growth stages: 14 days after anthesis (young) and 65 days after anthesis (ripe). Discs, 1.5 cm in diameter, were punched from the fruits and the cuticles isolated using an aqueous mixture of 2% (w/v) pectinase and 0.2% (w/v) cellulase buffered at pH 3.6. After 5 days of incubation at 30 °C the cuticles were recovered, extensively washed in deionized water, air-dried, and stored for further use. Cuticular waxes were removed by refluxing the isolated cuticles in chloroform:methanol (1:1) for 8 h.

Cutin samples were obtained after hydrolysis of dewaxed cuticles in a 6 M HCl solution for 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 (Luque et al., 1995). This series of exhaustive treatments to remove waxes and hydrolyzable compounds present in the isolated cuticles does not alter the chemical structure of the biopolymer (Walton, 1990). 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 sylanation with *N-O-bis*trimethylsilylacetamide, using a Hewlett–Packard 5890 GC-MS combination with a cross-linked methyl silicone capillary column.

2.2. FT-IR spectroscopy

KBr pellets were prepared using about 1.5 mg of cutin sample. Infrared spectra were recorded in a Perkin–Elmer 1760 Fourier-transform infrared spectrometer.

2.3. Solid-state ^{13}C NMR spectroscopy

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

2.4. Atomic force microscopy

AFM images were obtained with a Topometrix TMX2000 microscope operating either in contact constant force mode or in non-contact mode with both amplitude and phase detection. A large scale scanner (maximum X–Y range of 130 μm × 130 μm and 13 μm in Z) was used to analyze the overall texture and the homogeneity of the surface of cutin samples. When higher lateral and vertical resolution was needed another scanner with maximum X–Y–Z ranges of 2.3 μm × 2.3 μm and 0.8 μm was used. In any case, the same Si_3N_4 lever (contact NanoProbe, Digital Instruments, Santa Clara CA) with 0.58 N m^{-1} nominal constant force was employed. In non-contact mode, a stiff Si_3N_4 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

3.1. Chemical and spectroscopical characterization of isolated tomato fruit cutins

De-waxed cuticles from young and ripe tomato fruits after exhaustive acid hydrolysis yielded the polyester cutin; this fraction represented, in both cases, the 81% of the initial weight of the isolated cuticle. Moreover, for the two types of fruit cutins the major monomer found by GC–MS analysis was the 10,16-dihydroxyhexadecanoic acid. The amount of this fatty acid, in addition to the positional isomers, was 83.6 and 81.9% of the total weight of monomeric acids of young and ripe tomato cutins, respectively. This result agrees with others reported using different tomato varieties (Baker et al., 1982; Luque et al., 1995).

FT-IR spectroscopy can characterize in situ the functional chemical groups of isolated cuticles and their interactions with exogenous chemicals at the cuticular level (Luque et al., 1995; Ramírez et al., 1992). Fig. 1 shows the FT-IR spectra of young and ripe isolated tomato fruit cutins. The two spectra are very similar except for the presence of some absorptions around 1630, 1530, and 900–800 cm^{-1} for ripe sample. Such absorptions were not present in the infrared spectrum of the young tomato fruit isolate. Such absorptions are mainly assigned to the functional groups or structural characteristics of phenolics and flavonoids present in the cuticle and cutin of ripe tomato fruits. Thus, absorption around 1630 and 1550 cm^{-1} are assigned to the stretching of C=C bonds and the stretching of benzenoid rings, respectively. In addition, weak absorptions recorded between 900 and 800 cm^{-1} indicate the presence of di and tri substitutions in the aromatic rings. More details about these assignments can be found in some references (Luque et al., 1995; Ramírez et al., 1992; Villena et al., 2000). Nevertheless, in this case, it is interesting to evaluate the ratio between the two main infrared features that can be found in cutin material: the two strong bands located near 2900 cm^{-1} assigned to the asymmetric and symmetric stretching vibrations of the methylene group, the most repeated structural unit in the cutin biopolyester, and the strong absorption band at 1730 cm^{-1}, assigned to the C–O stretching vibration of the carbonyl group of the ester bond, i.e., the link between the different hydroxy fatty acids to form the cutin cross-linking. The ratio was lower (0.72) for cutin isolated from ripe tomato fruits than for the cutin obtained from young fruits (0.92). This is an indication of a higher cross-linking in the cutin of ripe tomato fruit cuticles. The weight per area unit measured for the two cutin isolates confirms this fact: 994 and 1528 $\mu g\,cm^{-2}$, for young and ripe cutin, respectively.

Solid-state NMR of the isolated cutins can provide useful structural information on the type of links that takes place in this solid matrix. Fig. 2 shows the ^{13}C NMR spectra of the two cutin samples investigated here. Main resonances assignments were as follows (Zlotnik-Mazori and Stark, 1988): $(CH_2)_n$, 29 ppm; CH_2CH_2OCOR, 64 ppm; *C*HOCOR and *C*HOH, 71.6 ppm; and CHO*C*OR, 173 ppm. The two NMR spectra were, again, very similar with the exception of the presence of additional resonances between 115 and 130 ppm in the NMR spectrum of ripe tomato cutin, a clear indication of the

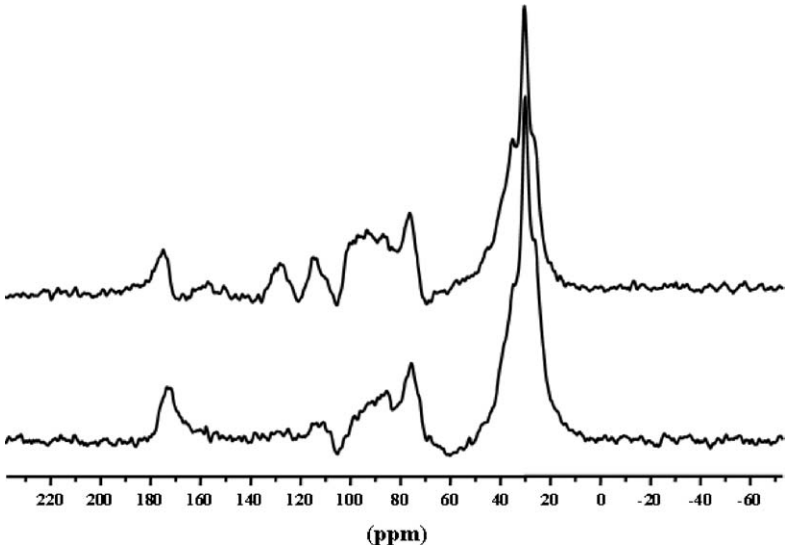

Fig. 1. ^{13}C CP/MAS NMR spectrum of the isolated ripe tomato fruit cutin (upper spectrum) and young tomato fruit cutin (lower spectrum). The main chemical-shift assignments are as follows: $(CH_2)_n$, 29 ppm; CH_2CH_2OCOR, 64 ppm; *C*HOCOR and *C*HOH, 71.6 ppm; and carbonyl, 173 ppm. For more details, see text.

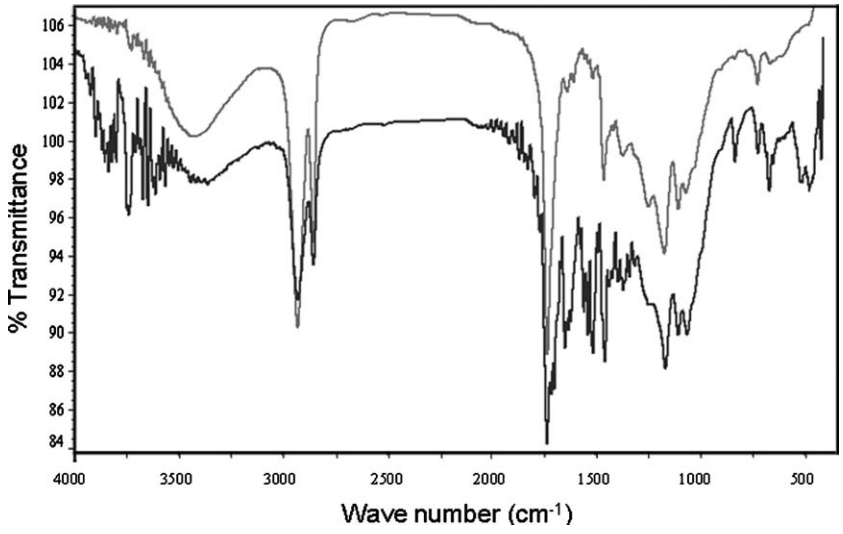

Fig. 2. Fourier-transform IR spectra for isolated ripe tomato fruit cutin (lower spectrum) and young tomato fruit cutin (upper spectrum).

existence of aromatic and unsaturated compounds. Another interesting molecular characteristic can be reached from ^{13}C NMR spectra: the above mentioned resonances indicate that the polyester of these isolates was mainly formed by esterification of secondary hydroxyl functional groups (173 ppm). However, this fact does not exclude the presence of ester links made from primary hydroxyl groups (chemical shift at 168 ppm; Zlotnik-Mazori and Stark, 1988), also present in the cutin network.

An indirect measure of the chemical mobility of the polymer chains that form the cutin could be given by the determination of the spin relaxation time, $T_{1\rho}$, of the $^{13}CH_2$ of cutin samples. This information directly regards the dynamics and structure of the biopolyester. The estimated $T_{1\rho}$ for young and ripe cutin were 118 and 58 μs, respectively. These values suggest a moderately rigid environment, more restricted dynamically in the case of ripe cutin, than the same motional segments in the cutin isolated from young fruits.

3.2. Microscopic characterization of isolated fruit cutins

The surface structure of tomato fruits cuticles and cutins is usually checked by scanning electron microscopy (SEM). Nevertheless, fine resolution cannot be achieved due to the gold layer (usually between 20 and 50 nm) that must be deposited on the sample surface. However, it is known that AFM has been used with success to image the surface morphology and elucidate structure details of amorphous and crystalline polymers and phase separated macromolecular systems.

Some aspects should be considered when studying tomato cutin samples by AFM. Among the operating modes of an AFM, the so-called "contact mode" is usually preferred because of its higher resolution. Besides, working in contact allows the mechanical and tribological study of materials, a quite important issue in cutins isolated from commercial fruits. However, when analyzing biological specimen, the relatively high forces applied in contact mode may cause some damage. However, no such damage has been reported in the literature (Round et al., 2000) when studying the mechanical characteristics of tomato fruit cutin in contact mode. Nevertheless, we have performed some wear tests by scanning small areas of cutin at high speed and under low and moderate loads and no erosion has been observed. Another option to prevent damage is to work in contact inside a liquid (typically water). This way, capillary forces are almost eliminated and scanning can be made under very low applied pressures. However, we have results showing a dramatic modification of mechanical properties of cutin when exposed to water. We suspect that such modification may be accompanied by an alteration of macromolecular arrangement of cutin structure. For this reason, working in liquid has been discarded in this work.

Another aspect to consider is the possibility of deforming the surface structure of cutin under contact. To address this point, we have analyzed the same cutin sample (and roughly the same spot) using both contact and non-contact modes. Results are shown in Fig. 3 and no texture modification can be appreciated. Moreover, the resolution of contact mode is significantly higher.

Cutin samples prior to de-waxing have also been analyzed and results are displayed in Fig. 4. Large range images are dominated by cross-linked rows approximately 0.5 μm wide. Some big agglomerates are also visible. A closer look reveals a background containing small patches of crystalline epicuticular waxes that prevents the direct observation of the cutin framework. Consequently, cutin samples had to be de-waxed.

AFM analysis of tomato fruit de-waxed cutin and free of hydrolyzable components revealed a well-defined surface texture that was not resolved by SEM (Fig. 5). Besides, AFM provides topographic data in the X, Y,

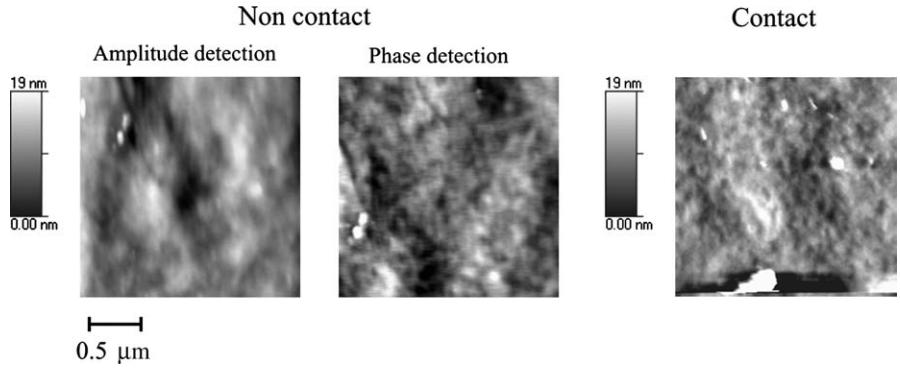

Fig. 3. Non-contact (both amplitude and phase detection) and contact high resolution AFM images obtained from the same ripe tomato cutin specimen. No damage or modification of surface but a better resolution is obtained if working in contact mode.

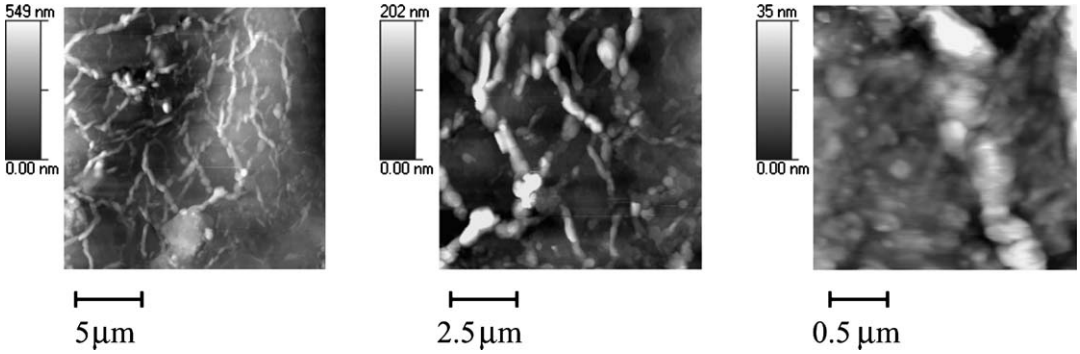

Fig. 4. Wide range AFM images showing the structure of waxes in tomato cutin prior to de-waxing. Characteristic interlaced rows about 0.5 μm thick as well as an amorphous background are observed.

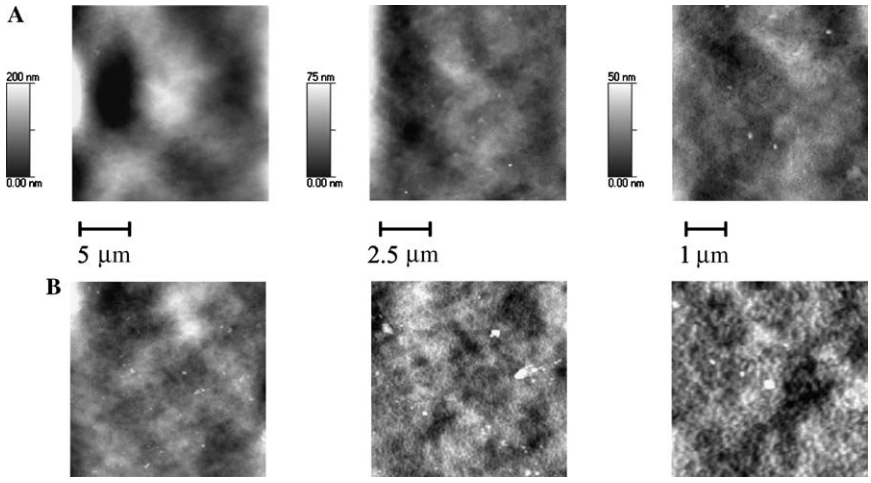

Fig. 5. AFM topographic images (contact mode) showing the morphology of the outer surface of isolated young (A) and ripe (B) tomato fruit cutin at different resolutions.

and Z directions (SEM only in X and Y). We would like to remark here some additional advantages of AFM compared with SEM in the analysis of biological tissues. First, no metallization of surface is necessary for AFM. In this sense, AFM is a non-destructive technique which is of great interest when unique, low yield or hard to prepare samples have to be studied. Thus, AFM provides the opportunity for nanometer scale, non-intrusive, three-dimensional imaging of cutin surface structure. Furthermore, the lack of surface metallization opens the possibility of analyzing surface texture changes induced by mechanical stress (as part of the study of the mechanical properties of biological tissues) and those produced upon exposure to controlled humidity. Also, thermal damage produced by a high energy electron beam and changes created by environment (drying,

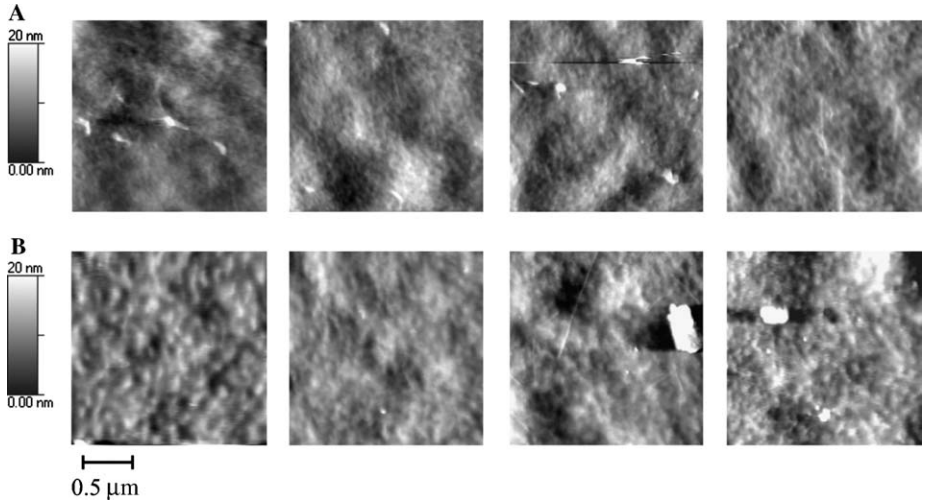

Fig. 6. High resolution AFM images of the outer surface of cutin isolated from (A) young and (B) ripe tomato fruits. Different spots in different samples are plotted to ensure reproducibility.

wrinkling, vesicle bursting, etc.) of a typical SEM preparation, are avoided.

Fig. 5 shows a series of wide range AFM images showing the homogeneity and typical textures of young and ripe tomato cutin. As observed, the topography of cutin from young fruits is modulated by soft and spaced wrinkles while cutin from ripened isolates appeared flatter.

Higher resolution images, Fig. 6, showed a characteristic short range texture that differentiates both type of cutin samples. Images corresponding to different spots in different samples have been displayed to show the reproducibility of our measurements. In the case of cutin isolated from ripened fruits, the surface can be described as an interlaced network of "worm-like" features spaced 200–300 nm. In some regions such features were randomly scattered but in others they appeared linked across a single direction giving rise to much more elongated structures. Also circular clusters were observed as the consequence of such a high interlacing. However, the cutin of young tomato presented a much less interlaced and more homogeneous texture. The size of the surface features in the X–Y plane is also two to three times smaller (about 70–100 nm).

The structural changes observed in the cutin surface of the samples investigated in the present work by AFM agree well to the cutin molecular characteristics and properties reached from spectroscopic tools and complete our current knowledge on this biopolymer. Thus, cutin isolated from tomato fruits appears as an amorphous polyester mainly formed by esterification of secondary hydroxyl groups of the dihydroxy fatty acids monomers with a different texture depending of the developmental stage of the fruit. This texture is a consequence of the actual status of the cross-linking degree of the polymer. The more dense and cross-linked cutin of ripe tomato is characterized by a flatter long range texture and by the development of elongated and oriented superstructures.

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