Nanostructure of natural and model cell wall materials**

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A b s t r a c t. Cell walls are the major component of plant tissue which significantly influence on textural properties of fruit and vegetables. Geometrical dimensions of parenchyma cells are very small what makes impossible studying their mechanical properties in the natural state. An alternative is creating of a model artificial cell wall consisting of polysaccharides which imitate properties of the natural cell wall. Polysaccharides network based on bacterial cellulose supplemented with xyloglucan and pectin was proposed as the model cell wall. In this experiment, the nanostructure of the model and the natural cell walls on the basis of their atomic force microscope topographs was compared in order to evaluate correctness of methodology of production of artificial cell walls. It was demonstrated that the bacterial materials have very similar artificial structure to the natural cell walls. However, considering the chemical composition, the most suitable as the model is the BCPX material consisting of bacterial cellulose, pectin and xyloglucan.

K e y w o r d s: nanostructure, cell walls, apple, model materials, atomic force microscope

INTRODUCTION

Cell walls are the major component of plant tissue which significantly influences on textural properties of fruit and vegetables (Jackman and Stanley, 1995; Vanstreels *et al.,* 2005; Waldron *et al.*, 2003; Zdunek, 2008). Geometrical dimensions of parenchyma cells are very small what makes impossible studying their mechanical properties in the natural state. An alternative is creating of a artificial cell wall consisting of polysaccharides which imitate properties of the natural walls (Astley *et al.*, 2003). The model material should characterize of chemical composition and microstructure which are adequate to the natural cell walls. Primary cell walls are mainly consisted of polysaccharides which constitute more than 90% of their dry mass. Polysaccharides of cell walls are cellulose (15-30% d.m.), pectin (about 30% d.m.) and hemicelluloses (about 25% d.m.) (Taiz and Zeiger, 2002).

Spatial structure of bacterial cellulose microfibrils which is produced by *Gluconacetobacter xylinus* is considered as very similar to the structure of the cellulose in the natural cell walls (Chanliaud *et al*., 2002). As a model material imitating natural cell wall polysaccharides network based on bacterial cellulose supplemented with tamarind xyloglucan and apple pectin was proposed (Chanliaud *et al.,* 2002; Cybulska *et al.*, 2010; Whitney *et al.*, 1995).

The cell wall structure analysis requires microscopes that allow observation in a nanoscale. Arrangement, dimensions and interactions between cell wall polysaccharides of different plant tissues were analysed by means of electron microscopy (McCann *et al.*, 1990). Electron microscope was used for observation of structural changes of fixed apple and pear cell walls which were exposed to enzymatic degradation simulating changes in cell walls during ripening. On the basis of microscopic images it was shown that changes of unripe tissue treated polygalacturonase are similar to changes which occur during natural ripening (Ben-Arie *et al.*, 1979). Scanning electron microscope was applied to evaluate the structure of different food products in order to relate the structural properties with a sensory analysis (Błaszczak and Fornal, 2008; Pierzynowska-Korniak *et al.*, 2004).

Sequential chemical analysis and drying of potato parenchyma cell walls showed that cellulose microfibril diameter depends on presence of pectin and hemicellulose on the surface of the microfibriles. Microfibril diameter in hydrated state which were not subjected to chemical treatment was on average 26.2 and 11 nm after drying and

extraction which caused removing of a soluble polysaccharides (Kirby *et al.*, 2006). Davies and Harris (2003) applied the atomic force microscope (AFM) to measure of cellulose microfibrils in partially hydrated sampled of model dicoyledon *Arabidopsis thaliana* and monocotyledon – onion. Diameter of cellulose microfibrils of *A. thaliana* were about 3.2 nm and of onion were 4.4 nm after extraction. According to the AFM studies of Thimm *et al.* (2000), cellulose microfibrils dimensions of celery varied in the range from 6 to 25 nm.

The AFM is widely used for biological applications. In AFM, the soft cantilever connected with a sharp tip is oscillated vertically over the surface and deflections of the tip are recorded (Engel *et al.*, 1999; Kirby *et al.*, 1996). Morris *et al.* (1997) applied AFM to observations of hydrated cell walls of chestnut, pectin from tomato tissue and carrageenan. Changes of arrangement of cellulose microfibrils of growing cells were also investigated by means of AFM (Marga *et al.*, 2005).

The aim of this research was to compare the nanostructure of the model cell walls with the nanostructure of the natural cell walls on the basis of atomic force microscope images in order to evaluate correctness of methodology of production of the artificial cell walls.

MATERIALS AND METHODS

In the experiment three different types of cell wall materials were compared: bacterial artificial cell walls, reconstituted apple cell walls and fixed apple tissue.

For the isolation of the cell wall material from apple tissue (*Malus domestica* Borkh. cv. Jonagored) fruit were used that were stored at 1°C and 95% relative humidity (RH) under controlled atmosphere (CA) conditions (1% O_2, 2.5% CO_2) prior to the isolation. Randomly selected fruits were used in the experiment. Apple fruit used for cell wall observations by means of atomic force microscopy were stored at 4°C under regular atmosphere.

Apple pectin, with a methylation degree at about 30% was purchased by Herbstreith and Fox (Neuenbürg, Germany). Xyloglucan from tamarind seeds (*Tamarindus indica* L.) was purchased from Megazyme (Bray, Ireland).

Bacterial artifical cell walls materials were produced using the protocols described by Cybulska *et al.* (2010). Briefly, bacteria strain *Gluconacetobacter xylinus* was cultured in liquid Hestrin Schramm culture medium (HS) containing: glucose (2%), bactopeptone (0.5%), yeast extract (0.5%), disodium phosphate (0.27%), and citric acid (0.115%). Acidity (pH) was adjusted to 5.0 with 5M NaOH (Hestrin and Schramm, 1954). The bacterial artificial cell walls were grown on the top of the medium. Depending on the medium composition different artificial cell walls were obtained:

– BC – bacterial cellulose,
– BCP – bacterial cellulose embedded in pectin (BCP) obtained by adding apple pectin to the medium up to 5 g l^{-1} and 12.5 mM calcium chloride prior to sterilization,

– BCPX – bacterial cellulose with pectin and xyloglucan created by adding the apple pectin (2.5 g l^{-1}), xyloglucan (2.5 g l^{-1}) and 6 mM calcium chloride to the culture medium, prior to sterilization.

The temperature of incubation was 28°C for BC, BCP and BCPX. Thin films of BC, BCP and BCPX were collected 7 days after inoculation. Films of pure bacterial cellulose (BC) were washed many times in distilled water, and in the case of materials containing pectin (BCP and BCPX), in 12.5 M solution of calcium chloride. The materials were stored in 0.02% sodium azide solution at 4ºC prior to testing to prevent degradation.

Apple call wall material was isolated using modified phenol-buffer method proposed by Renard (2005). Frozen apple slices were homogenised in a cool buffer simulating the ionic conditions in apple juice (1.2 mM $CaCl_2$, 2.0 mM $MgCl_2$, 0.5 g l^{-1} KCl, 60 mg l^{-1} ascorbic acid, 4 g l^{-1} malic acid, 1 g l^{-1} sodium disulfite supplemented to pH 3.5 with 5 M NaOH) with Triton 100 (2 g l^{-1}) and 1-octanol (4 ml). The suspension was then filtered under reduced pressure and washed in 60% water solution of acetone. The resultant paste was blended with phenol at a volumetric ratio of 1:4 and left for one hour at room temperature. Next, the blend was dissolved in the buffer and filtered. The material was washed in 70 and 96% ethanol, and finally in acetone. The sample was vacuum dried.

Specimens of apple parenchyma tissue (approx. 10 mm long, 2 mm wide and 2 mm thick) were sliced by hand using a scalpel. The specimens were fixed under reduced pressure by means of the mixture of glutaraldehyde, acetic acid and formalin. After 24 h of fixation, the specimens were rinsed in phosphate buffer (pH 7.0) and then dehydrated in a serie of ethanol solutions (5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 and 96%) and finally thrice in acetone. Dehydrated specimens of apple tissue were dried at the critical point.

Model polysaccharide networks (BC, BCP, BCPX) of apple cell wall material, pectin and xyloglucan used in the experiment were subjected to analyse of chemical composition. Total uronic acid content was measured by colorimetry using galacturonic acid as a standard (Cybulska *et al.*, 2010). Quantification of neutral sugars was performed by gas-liquid chromatography (GC) after sulfuric acid degradation and derivation as alditol acetates (Delcour *et al.*, 1999). Sugars were identified and quantified by comparison with reference sugars.

To estimate the amount of cellulose, samples were pre-hydrolyzed in sulfuric acid at room temperature for 3 h and then after addition of water, samples were hydrolyzed at 100°C for 2 h and further treated as described above. The amount of glucose originating from cellulose was calculated as the difference between the amount of glucose determined in the procedure with pre-hydrolysis and amount of glucose determined in the procedure without pre-hydrolysis.

All the cell wall materials produced (BC, BCP, BCPX, CWMR, FAT) were subjected to observations by means of the Atomic Force Microscope (NanoScope III, Digital Instruments, Veeco Metrology Group, Dourdon, France). Prior to the microscopic observations the dried specimen were stored in airtight containers to prevent rehydration. Dried sampels were glued to carbon tape and subjected to AFM observations.

AFM images were analysed quantitatively by means of WSxM 4.0 Develop 8.0 software (Nanotec, Electronica). The software is designed for scanning probe microscopy including atomic force microscopy (Horcas et al., 2007). A diameter of microfibrils was determined on the basis of the two-dimensional height mode images. Linear sections were marked out randomly on the images. Then sections were automatically converted into height profiles. On the gained profiles width of peaks was measured in the way given on the Fig. 1. It was assumed that the each peak represents one cellulose microfibril. Mean diameter of microfibrils was determined on the basis of 50 measurements from 10 images for the each material.

RMS (root mean square) roughness, ironed surface, maximum height and average height were determined automatically for the each AFM image of the model and the natural apple cell walls.

Roughness of image surface analysis was carried out automatically on the basis of histograms which performed distribution of the height of structural elements from 2D height images (Fig. 2).

To determine RMS roughness, height and ironed surface, 2 images of the each material: CWMR, 1 of FAT, 6 of BCPX, 1 of BC and 2 of BCP were taken. The size of the images was 2x2 µm. Statistical analysis using STASTICA® 8.0 (StatSoft, Inc. 1984-2008) was performed. Post-hoc HSD Tukey test for unequal numbers was used for determination of significant differences among materials.

RESULTS

The method of sample preparation affects the microstructure of obtained materials. In the case of the natural cell wall, two methods of sample preparation to AFM were applied. One of them was the isolation of cell wall material from apple parenchyma tissue (CWMR material). The second method was the fixation of apple tissue according to preparation typical of light microscopy and then application of drying in critical point (FAT material). Because of the fact that the second method has not been applied to apple tissue yet, the images of samples prepared with both methods were compared (Fig. 3). In both cases quality of images was good enough to qualitative and quantitative analysis.

Figure 3a shows AFM image of the apple cell wall material isolated with the buffer-phenol method (CWMR). Long cellulose microfibrils creating a network are clearly visible in the picture. Microfibrils are situated in chaotic manner. Fig. 3b presents fixed apple tissue (FAT). Prepara-
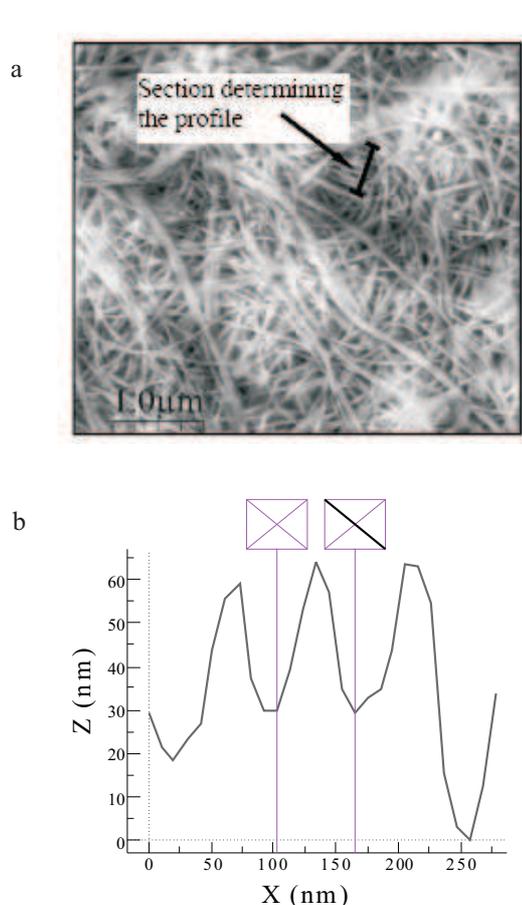

Fig. 1. Measuerement of the cross-section diameter of single microfibril: a – AFM picture of bacterial cellulose with linear section used for diameter measurements, b – determination of the microfibril diameter from the section profile.
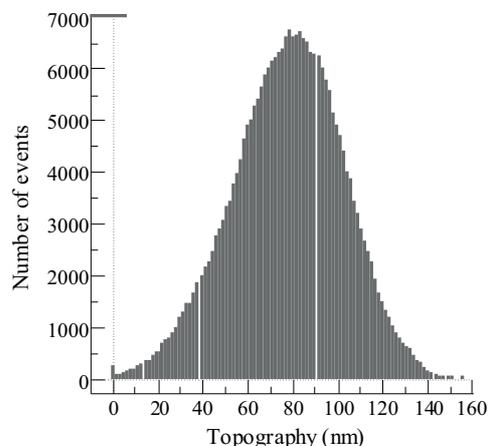

Fig. 2. Histogram of AFM image of bacterial cellulose microfibrils obtained by means of WSxM 4.0 Develop 8.0 software.
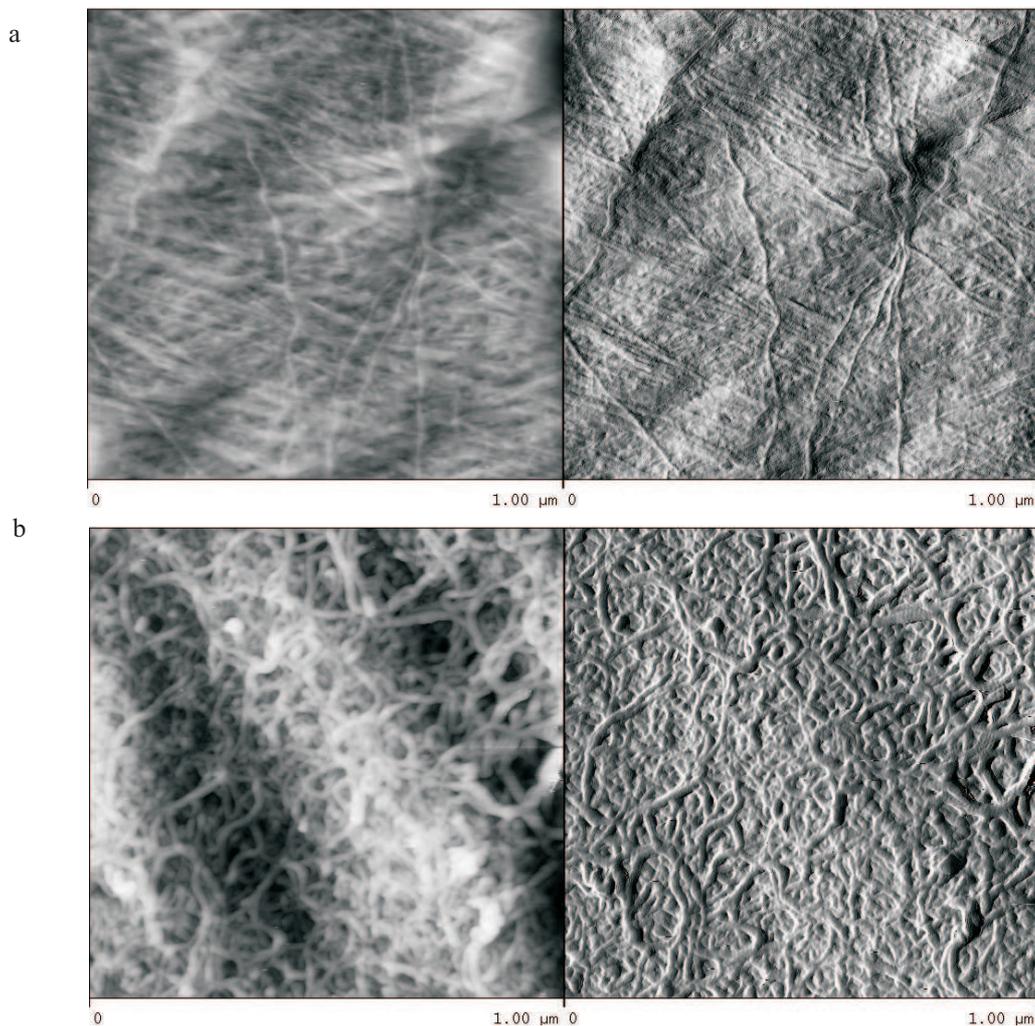
Fig. 3. Topography of AFM images 1x1μm, height mode (left) and amplitude mode (right): a – reconstituted apple cell wall material prepared by means of the buffer-phenol method (CWMR), b – fixed apple cell wall (FAT).

tion of apple tissue by means of the buffer-phenol method (CMWR) results in powder of very small cell wall particles. Microfibrils on the surface of CWMR material were thinner and relatively straight, without tangles. This lead to a conclusion that probably microfibrils were straightened due to scouring off a part of cross-linking substances like hemicelluloses, ions or protein (Fig. 3a). More wavy structure of the fibrils of FAT material (Fig. 3b) can be an effect of using ethanol as a component of fixing mixture. Ethanol causes shrinking tissue cells and it can effect deformation of cellulose microfibrils.

Bolder cellulose microfibrils in fixed apple cell wall (FAT) in comparison with CWMR material (Fig. 3) is probably combined with the fact that preparation did not result in pectin and hemicellulose removing from the surface of

cellulose microfibrils. As a result, microfibrils were surrounded by pectin and hemicellulose networks what had an effect of their thickening. Determination of approximate length of cellulose microfibrils was difficult in case of FAT due to the spatial structure of the materials. Microfibrils of FAT are less visible and they are blocked by pectin network to a higher degree than cell walls isolated by means of the buffer-phenol method (CWMR). The length of visible elements of cellulose microfibrils of FAT is very roughly about 360 nm. Bringing out of individual fibrils in the surface of particles is an advantage of apple cell wall isolation by means of the buffer-phenol method (CWMR) because it enables to estimation of the length of microfibrils. The length of visible fragments of microfibrils CWMR material was estimated about 700 nm.

Chemical composition analysis of CWMR demonstrated that pectin content was approx. 26%. However, Voragen *et al.* (2001) reported that pectin content in apple tissue is about 40%. This difference is probably caused by depolymerization of a part of pectin chains during preparation regardless maintaining pH at the level of about 3.5. In this rage of pH, pectic compounds could be depolymerised by their hydrolysis. Moreover alkali (sodium hydroxide) and alcohol used for cell wall material isolation destabilised glicosidic bonds and simplified pectin depolymerization. The effect of partial pectin chains disintegration took a place on the surface of cell wall particles which had bigger contact surface with chemical substances than pectin inside the particles, in deeper layers of cellulose network. Fig. 3a and Fig. 3b show cellulose microfibrils on the surface and more compact structure in the background. Cellulose fibrils seem to be submerged in the matrix which contain large amount of pectin inside particles.
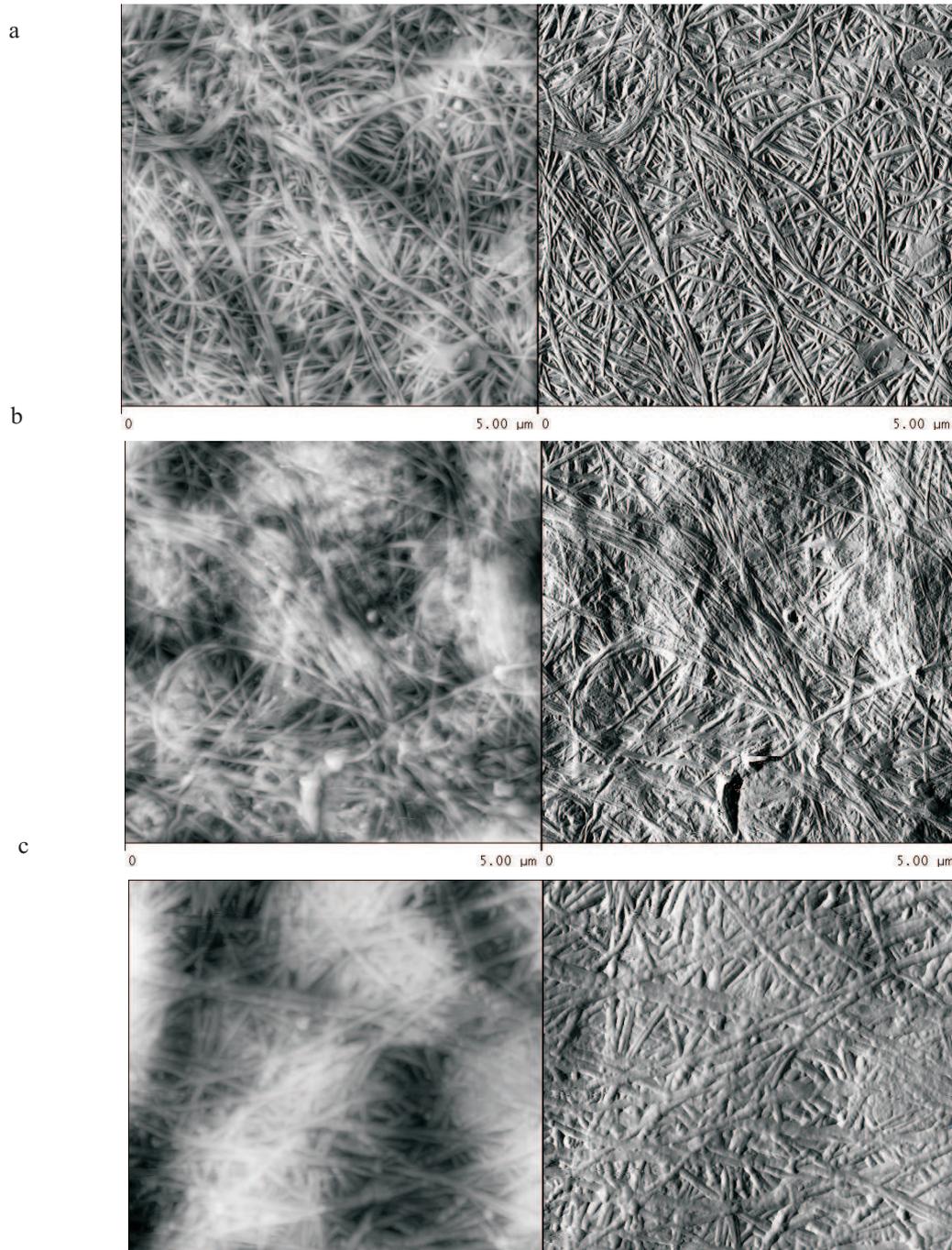

Fig. 4. Height (left) and amplitude (right) mode AFM topographs of bacterial model polysaccharide networks, 5x5 μm images: a – BC, b – BCP, c – BCPX.

Model bacterial cell walls in dry state were also subjected to AFM observations (Fig. 4). All the pictures presented are in the same scale in order to simplify their qualitative comparison. The materials had different chemical composition and this difference was reflected in the microstructure. Pure bacterial cellulose (BC) characterized the most porosity structure in comparison with the other materials. Any matrix was not visible but only chaotically located network of branched microfibrils. Cellulose microfibrils were arranged mainly randomly but there are also some bundles of several microfibrils. Similar bundles were observed in AFM images of chestnut cell walls (Kirby *et al.*, 1996). Their presence is probably an evidence of crystalline structure of cellulose microfibrils.

After adding pectin to culture medium (BCP) porosity have significantly decreased, there were only a few small pores. In BCP material containing bacterial cellulose (approx. 12%) and pectin (approx. 78%) only chemical analysis evidence the presence of pectin in matrix. In this material, bundles of cellulose microfibrils can be also observed but observation is difficult due to embedding in matrix. However, significant thickening of cellulose microfibrils was not ascertained in BCP material.

In the three-component composite (BCPX, Fig. 4c) containing about 26% cellulose, 44% pectin and 20% xyloglucan, microfibrils are considerably thicker in comparison with BC and BCP and no distinct clustering in bundles was observed. It is probably xyloglucan effect which creates strong bonds with cellulose microfibrils. Cellulose microfibrils might be easily coated with xyloglucan (Cosgrove, 2005) what can cause their thickening and modification of cellulose structure making it similar to plant cellulose (Atalla, 1999). These observations lead to conclusion that pectin network is weaker connected with cellulose microfibrils than hemicellulose network.

AFM topographs of model polysaccharides networks and apple cell wall material were subjected to quantitative analysis. Mean diameter of cellulose microfibrils was determined for 50 subjectively chosen microfibrils from 5 images of each material (Fig. 5). Diameter of cellulose microfibrils was different for studied materials. For apple cell wall materials, diameter of cellulose microfibril amounts about 13 nm for CWMR or 22 nm for FAT. This difference is probably the effect of different chemical preparation in the methods. Isolation of cell wall material by means of phenol-buffer method (CWMR) caused removing of pectin and hemicelluloses from the surface of material particles, what induced exposition of cellulose network and decrease of cellulose microfibrils in AFM topographs. However in fixed tissue (FAT), pectin and hemicelluloses were not removed from its surface and stayed connected with cellulose microfibrils what caused their larger thickness.

Diameters of cellulose microfibrils of primary cell wall reported in literature vary from several to several dozen nanometers. Thimm *et al.* (2000) determined diameters of cellulose microfibrils as from 6 to 25 nm on the basis of AFM images of celery cell walls in the natural state without drying. The diameter of microfibrils of carrot and potato cellulose was determined as on average 25 nm and for water chestnut 48 ± 10 nm (Kirby *et al.*, 1996). McCann *et al.* (2001) quantified a thickness of cellulose microfibrils after CDTA (1,2 cyclohexane-diaminetetra-acetic acid, Na salt), sodium carbonate and potassium hydroxide extraction as 8 nm on the basis of electron microscope images. Taking into consideration the dimensions determined in the experiment by means of the two methods: buffer – phenol and fixation of tissue it can be assumed that they are related to the dimensions estimated in previous research.

Diameter of bacterial cellulose microfibril in BC material amounted approximately 37 nm. It is comparable with the data presented in another researches. Tokoh *et al.* (2002) after analysis of electron microscope images reported that width of bacterial cellulose microfibril amounts about 43 nm. Dammstrom *et al.* (2005) determined diameter of bacterial cellulose microfibrils as 60-100 nm from AFM images. Whitney *et al.* (1995) determined the same as 36 nm on the basis of images from transmission electron microscope whereas Chanliaud and Gidley (1999) as 24-86 nm. The differences between results in this experiment and reported in other studies can relate to type of bacteria strain, time of incubation, stirring and microscope used to observations.
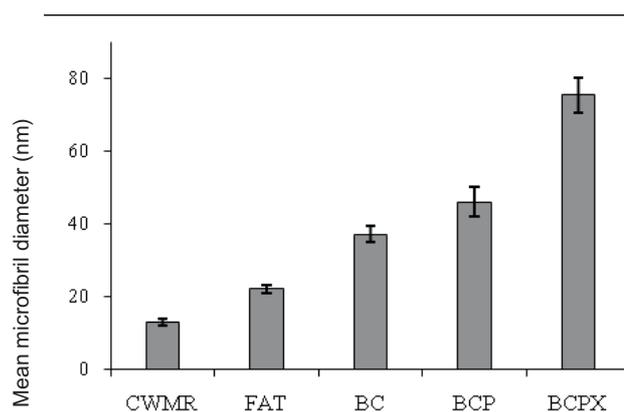

Fig. 5. Mean diameters of cellulose microfibrils of the model materials and the apple cell wall materials. CWMR – apple cell wall material isolated from apple parenchyma, FAT – fixed apple tissue, BC – bacterial cellulose, BCP – bacterial cellulose with pectin, BCPX – bacterial cellulose with pectin and xyloglucan. The bars show confidence intervals at confidence level $\alpha = 0.05$.

T a b l e 1. RMS roughness, mean height, maximal height and ironed surface of AFM topographs of apple cell walls and model cell walls

Material	RMS roughness	Mean height (nm)	Maximal height (nm)	Ironed surface (μm^2)
CWMR	13.1[a]	49.0[a]	116.7[a]	5.0[a]
FAT	36.6	148.8[a]	299.4	7.3
BC	14.8[a]	58.7[a]	103.8[a]	5.5[a]
BCP	16.0[a]	68.2[a]	125.3[a]	4.9[a]
BCPX	12.0[a]	74.2[a]	131.2[a]	4.4[a]

CWMR – apple cell wall material isolated from apple parenchyma, FAT – fixed apple tissue, BC – bacterial cellulose, BCP – bacterial cellulose with pectin, BCPX – bacterial cellulose with pectin and xyloglucan. [a]The same letter in a column means no significant difference at $p < 0.05$.

Addition of pectin to the culture medium (BCP) caused thickening of bacterial cellulose microfibril to about 46 nm. However, xyloglucan supplementation (BCPX) had the most significant influence on microfibril dimensions which caused increase of diameter to about 75 nm. In this case, the increase of microfibrils thickness was probably connected with covering of microfibrils with xyloglucan monolayer, what is consistent with hypothesis of primary cell wall model (Fry, 1989; Hayashi *et al.*, 1994; Levy *et al.*, 1997).

RMS (root mean square) roughness, mean and maximal height of profile and ironed surface of profiles are shown in Table 1. The data shows that roughness was similar for CWMR and bacterial artificial cell walls. However fixed apple tissue (FAT) had significantly bigger roughness of profile in comparison with the other materials. It can indicate that on the surface there was a big amount of structural elements or their dimensions were bigger. On the basis of diameter analysis (Fig. 5) it was claimed that the diameter of cellulose microfibrils of fixed apple tissue (FAT) was significantly lower than bacterial materials so larger concentration of microfibrils could decided about bigger roughnes. Slight shrinking of FAT was observed. It was the effect of preparation which caused decrease of the distance between microfibrils and caused their concentration what can be an explanation of considerable difference in roughness between FAT and the other materials.

Similarly as in the case of roughnes, mean and maximal height of profile were the largest for FAT. The other materials did not differ significantly ($p>0.05$). Mean and maximal height of profile are almost two times larger than fixed apple tissue.

There were no significant differencies of ironed surface between BC, BCP, BCPX and CWMR. For fixed apple tissue ironed surface was larger but the difference is not so distinct as in the case of roughness or the height of profile. Larger ironed surface for fixed apple tissue was caused by larger amount of microfibrils on certain surface.

CONCLUSIONS

1. Microstructural dimensions and microfibrils arrangement were mostly affected by sample preparation procedure; by both: material production procedure and sample preparation protocol for AFM observation.

2. Material obtained by fixation of apple tissue (FAT) had significantly higher topographical parameters comparing to the artificial (BC, PCP, BCPX) and isolated cell walls material (CWMR) with exception of microfibrils diameter which was similar to these materials.

3. All the bacterial materials obtained in the experiment demonstrated some similar features of their artificial structure to the natural cell walls (CWMR), however considering the chemical composition, the BCPX material consisting of bacterial cellulose, pectin and xyloglucan is the most suitable as the model of cell wall.

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