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Temporal Sequence of Cell Wall Disassembly Events in Developing Fruits. 2. Analysis of Blueberry (*Vaccinium* Species)

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Softening and pathogen susceptibility are the major factors limiting the marketing of blueberries as fresh fruits, and these traits are associated with fruit cell wall structure. However, few studies that characterize wall modifications occurring during development and ripening have been reported for this fruit. In this study the ripening-associated modifications of blueberry fruit cell walls (composition, pectin and hemicellulose solubilization, and depolymerization) at five stages of ripeness have been analyzed. Xylose was found to be the most abundant noncellulosic neutral sugar associated with fruit walls, and the observed high Xyl/Glc ratio suggested that xylans, which are usually a minor hemicellulosic fruit wall component, are abundant in blueberry. The pectic matrix showed increased solubilization at early and intermediate stages of ripening, but no changes were detected in late ripening. Furthermore, little reduction in pectin polymer size occurred during blueberry ripening. In contrast, hemicellulose levels decreased as ripening progressed, and a clear depolymerization of these components was observed. A model for cell wall degradation in this fruit is discussed.

KEYWORDS: Blueberry; *Vaccinium* sp.; cell wall degradation; pectin; hemicellulose

INTRODUCTION

Blueberries are members of the Ericaceae family, genus *Vaccinium (1*). The fruit is an epigynous or "false" berry derived from an inferior ovary, unlike true berries that derive from superior ovaries (*1*). Consumer consciousness of food nutritional value has increased in the past few decades, and this awareness has increased the popularity of blueberries. Blueberry ranks high in antioxidant activity among fresh fruits (*2*), and it has been shown that dietary supplementation with fruit extracts may decrease the enhanced vulnerability to oxidative stress that accompanies aging (*3*). These beneficial properties might have contributed to a rapid increase in blueberry demand in recent years. Production has more than doubled since the late 1970s (*4*). To meet growing consumer demand, commercial blueberry acreage in the United States increased more than 60% between 1990 and 2005 (*4*).

Fresh blueberries are well-accepted by consumers, but their high perishability has meant that a significant proportion of fruit production is destined for processing, including jams, yogurts, sauces, juices, and other products. Rotting diseases are the greatest cause of postharvest losses in fresh berries. Thus, as for most fruits, firmness is a very important trait for preserving blueberry quality and shelf life and for consumer acceptance. The increasing conversion from hand- to machine-harvesting of blueberries, particularly by growers with large plantings or co-operatives, is another issue that could increase the demand for developing varieties with improved textural properties. Increased fruit popularity has increased shipping distances in the fresh blueberry market, and this may also affect the demand for fruits with reduced softening and, hence, increased shipping and shelf life (*5*). The biochemical characterization of cell wall polymers and their progressive modification during ripening have been studied in several fruits (*6*). However, only a very few studies have attempted to characterize the cell wall disassembly process in blueberry. In this work we have analyzed the modifications in blueberry cell wall structure and composition at five different ripening stages to understand cell wall disassembly in this perishable but highly appreciated fruit.

MATERIALS AND METHODS

Plant Material. Blueberry fruit cv. Duke was harvested at five different stages: large green (G), 25% surface blue color (25%B), 75% surface blue color (75%B), 100% surface blue color (100%B), and blue ripe (BR).

Firmness Measurement. Firmness was measured using a texture analyzer (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY) fitted with a 2 mm flat probe. Each fruit was compressed 2 mm at a rate of 0.5 mm s^{-1}, and the maximum force in newtons developed during the test was recorded. Eighty fruits were used for each stage analyzed.

Isolation of Cell Walls. Fruit cell walls were isolated according to the method of Vicente et al. (*7*). Thirty grams of fruit tissue for each

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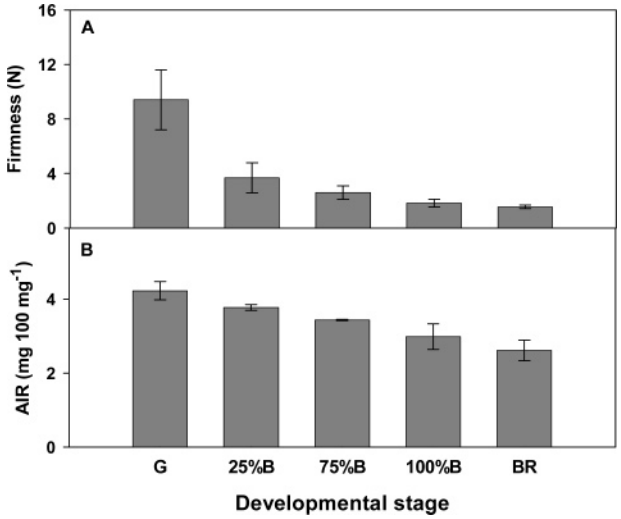

Figure 1. Changes in blueberry fruit firmness (**A**) and alcohol insoluble residue (AIR) (**B**) during ripening. G, green; 25%B, 25% surface blue color; 75%B, 75% surface blue color; 100%B, 100% surface blue color; BR, blue ripe. For fruit firmness 80 measurements at each developmental stage were done. AIR measurements were done in triplicate. The standard deviations are shown.

developmental stage was used to obtain the alcohol insoluble residue (AIR). The AIR was dried and weighed. Results were expressed as milligrams of AIR per 100 mg of fresh fruit.

Uronic Acids (UA) and Neutral Sugar (NS) Measurements. Three milligrams of AIR was solubilized in H_2SO_4 (*8*), and aliquots of the AIR solution were subsequently assayed colorimetrically for UA (*9*) and total sugars (*10*). Three independent samples were analyzed for each developmental stage, measurements were done in duplicate, and results were expressed as micrograms of galacturonic acid or glucose per milligram of AIR.

Cell Wall Fractionation. Fractions of different cell wall components were obtained by sequential chemical extraction of the cell wall material (AIR) according to the method of Vicente et al. (*7*). Two independent serial extraction series were performed for each developmental stage analyzed. Samples were assayed in triplicate for NS and UA as described above.

Size Exclusion Chromatography (SEC). The water (WSF), CDTA (CSF), Na_2CO_3 (NSF), 4%KOH (4KSF), and 24%KOH (24KSF) soluble fractions were chromatographed as described in Vicente et al. (*7*).

GC-MS Analysis. Dried samples from the WSF, CSF, NSF, 4KSF, and 24KSF or 2 mg of AIR for total cell wall analysis were hydrolyzed in 2 N trifluoroacetic acid (*11*) and converted to alditol acetates (*12*) for gas chromatographic analysis of neutral sugar composition. Measurements were done according to the method of Vicente et al. (*7*).

Statistical Analysis. Experiments were performed according to a factorial design. Data were analyzed using ANOVA, and the means were compared by the LSD test at a significance level of 0.05.

RESULTS AND DISCUSSION

Fruit Firmness, Cell Wall Yield, and Composition. Both fruit firmness and cell wall yield decreased as blueberry ripening progressed (**Figure 1**). The largest reduction in fruit firmness was observed from the green to the 25%B stages, a period in which fruit expansion and ripening were coincident. Interestingly, no great differences were observed in firmness between the 100%B and BR fruits. Colorimetric analysis of the cell wall's content of uronic acids, representing the main pectin constituents, and neutral sugars indicated that no overall changes in these general components occurred throughout ripening (**Table 1**). Specific analysis of the cell wall noncellulosic neutral sugar composition identified Xyl and Ara as the most abundant components in blueberry (**Table 1**). Xyl is not usually a very abundant sugar in fruit cell walls. In the primary cell wall of dicots and non-commelinoid monocots it is primarily associated with xyloglucan polymers. However, the fact that Xyl largely exceeds the Glc content suggests the presence of xylan in blueberry fruit cell wall.

Pectin Solubilization, Depolymerization, and Composition. Pectin in both the WSF and CSF increased between the green and 75%B stages (**Figure 2**). This increased solubilization was associated with a reduction of uronic acids in the NSF. However, after the 75%B stage, no further shifts were seen in the uronic acid contents of the three pectin-rich fractions. Ara was the most abundant neutral sugar component of the pectin fractions (**Figure 3**). The specific sugar content data provided by the GC-MS analysis revealed changes in the sugar compositions of the different pectin fractions, and the clearest changes were in their Ara and Glc proportions. Ara increased in the WSF and decreased in the NSF. The Glc in the pectin fractions was found primarily in the WSF and CSF, and the decrease that accompanied fruit ripening (mole percent) was confined to the WSF. In general, pectin size distributions differed between the three pectin fractions, with the smaller polymers found in the NSF, but there were no clear pectin size shifts in any of the fractions as ripening proceeded (**Figure 4**). The only exception was a slight decrease in size of the CSF at the BR stage.

The extent of pectin polymer breakdown has been shown to be variable among fruits (*6*). In some cases extensive changes in pectin size are observed, for example, as in avocado and peach (*13*, *14*). Tomato pectin depolymerization occurs to a lesser extent, but it is still substantial (*13*) relative to that in blueberries. Only a few fruits such as peppers, banana, and apple have been shown to undergo minor changes in pectin molecular size (*6*). Early reports also showed only a relatively modest decrease in CSF size in strawberry (*15*). However, recent studies on strawberry reported that the depolymerization of dilute HCl-soluble pectin, a pectin extract roughly equivalent to the NSF pectin depicted in **Figure 4**, can be substantial (*16*). Furthermore, Jiménez-Bermúdez et al. (*17*) reported reduced softening in strawberries with antisense suppression of genes encoding pectin lyase, an enzyme involved in pectin depolymerization. Thus, blueberry seems to be uncommon in that fruit softening occurs without substantial modifications in pectin molecular size. Highbush blueberry firmness has been improved by postharvest applications of calcium chloride (*18*), suggesting a role for the pectin matrix in determining fruit textural properties. Because the results from the analysis reported here show that ripening-associated softening in blueberry occurs without substantial pectin depolymerization, the positive effects of calcium on firmness retention may be caused by a reduced solubilization of pectin due to the formation of calcium bridges or an indirect effect on hemicellulose disassembly rather than prevention of pectin depolymerization.

Hemicellulose Solubilization, Depolymerization, and Composition. A reduction in the wall content of the 24KSF was observed during blueberry ripening (**Figure 5**). A slight increase in the amount of the 4KSF was observed over the same development period. The clearest modifications occurred by the time the 100%B stage was reached, with no subsequent changes in amounts of KOH-soluble polymers observed. Whereas the overall NS composition data for the 4KSF and 24KSF could be interpreted as reflecting a loss of 24KSF polymers and their appearance in more soluble form (i.e., in the 4KSF), the data for the specific neutral sugar composition of the two hemicel-

Table 1. Changes in the Content of Blueberry Cell Wall Uronic Acids, Neutral Sugars, and Noncellulosic Neutral Sugars during Ripening (Standard Deviations Are Shown)

	green	25% blue	75% blue	100% blue	blue ripe
UA (μg mg^{-1})	344 ± 14	329 ± 28	311 ± 12	314 ± 4	323 ± 14
NS (μg mg^{-1})	593 ± 23	616 ± 56	572 ± 35	536 ± 51	535 ± 42
Rha (mol %)	1.03 ± 0.03	1.04 ± 0.37	1.16 ± 0.08	1.15 ± 0.17	1.21 ± 0.06
Fuc (mol %)	ND	0.19 ± 0.02	0.07 ± 0.08	ND	ND
Ara (mol %)	26.66 ± 2.02	34.99 ± 2.31	31.93 ± 3.21	30.00 ± 3.56	30.75 ± 1.34
Xyl (mol %)	58.27 ± 1.94	44.78 ± 1.38	48.05 ± 3.45	48.58 ± 3.31	50.50 ± 2.91
Man (mol %)	0.86 ± 0.06	1.31 ± 0.08	1.17 ± 0.08	1.46 ± 0.09	1.31 ± 0.12
Gal (mol %)	7.15 ± 0.32	9.97 ± 0.76	9.52 ± 0.65	10.65 ± 0.65	9.39 ± 1.29
Glc (mol %)	6.03 ± 0.43	7.72 ± 1.66	8.10 ± 0.80	8.16 ± 0.21	6.84 ± 0.55

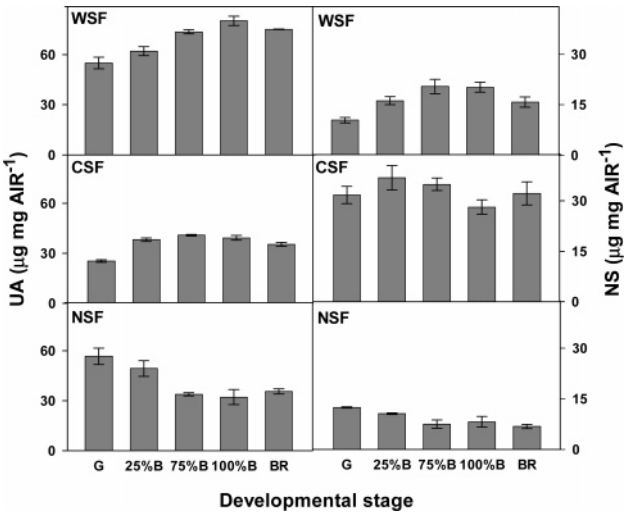

Figure 2. Changes in content of uronic acids (left) and neutral sugars (right) in the WSF, CSF, and NSF throughout blueberry fruit development. Ripening stages are defined as in the caption of **Figure 1**. Two independent extractions were done for each developmental stage, and samples were measured in triplicate. The standard deviations are shown.

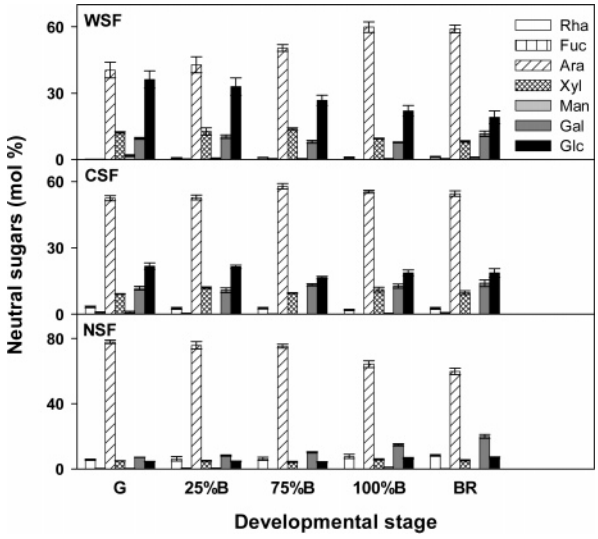

Figure 3. Neutral sugar composition (mole percent) of blueberry WSF, CSF, and NSF throughout fruit development. Ripening stages are defined as in the caption of **Figure 1**. Two independent extractions were done for each developmental stage, and samples were measured in duplicate. The standard deviations are shown.

lulosic fractions argue against this interpretation. Xyl was highly abundant in the 4KSF, and Ara was also a prominent component (**Figure 6**). This likely indicates the presence of glucouronarabinoxylans, which have a backbone of (1−4)β-D-xylan, with side chains of single units of nonreducing terminal α-L-ara and α-D-glcA. The main ripening-associated change in the 4KSF was a clear reduction in Ara content between the 75 and 100%B stages (**Figure 6**). This correlated with the increase in Ara content of the WSF (**Figure 3**). In the 24KSF, Glc and Xyl were the most abundant components.

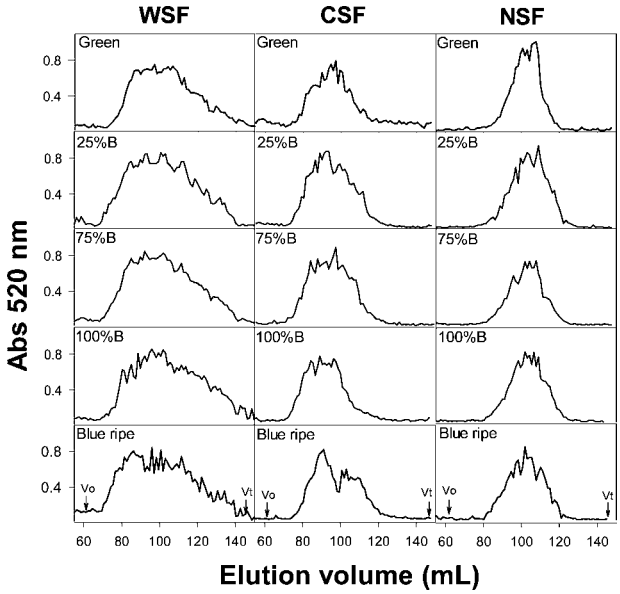

Figure 4. Size exclusion chromatography profiles from blueberry WSF, CSF, and NSF throughout ripening, fractionated on HW65. Column fractions (2 mL) were assayed for UA content using the *m*-hydroxybiphenyl method (*8*). Vo, void volume; Vt, total volume.

High Xyl content was recently reported in bilberry fruit, which also belongs to the genus *Vaccinium* (*19*). The bilberry seeds were particularly rich in Xyl, but the pulp cell walls still contained a relatively high proportion of this sugar. However, in bilberry the high level of Xyl was identified along with an even greater content of Glc, most likely supporting the presence of xyloglucans. The Xyl/Glc ratio observed for the 24KSF from the G and 25%B blueberry ripening stages (**Figure 6**) matches the 3:4 ratio expected for xyloglucan, which has been shown to be the predominant hemicellulosic polymer present in the strong base-extractable hemicellulose fractions of many fruits (*6*). The clearest ripening-associated sugar component change in the 24KSF was a relative decrease in Glc content that was first seen at the 75%B stage. The simplest explanation for the decrease in 24KSF Glc content is that the fraction contains a minor glucan component that is being digested. However, in late ripening (100%B to BR) the fraction's content of Xyl exceeds that of Glc, suggesting that the 24KSF contains a mixture of xylan and xyloglucan and that, as ripening continues,

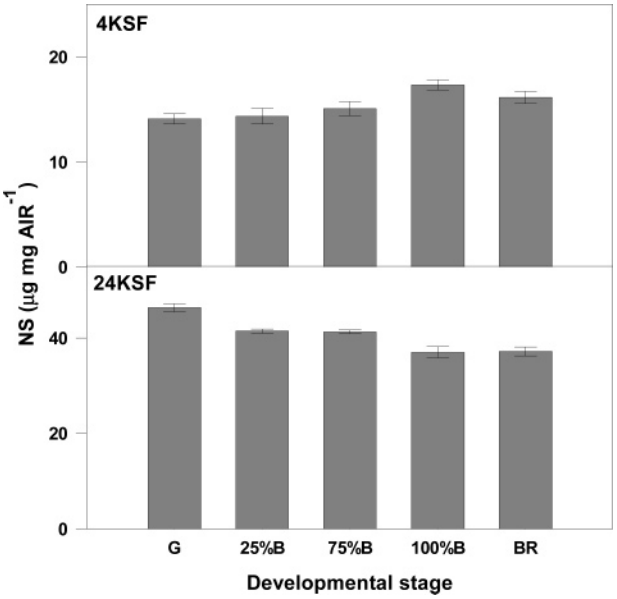

Figure 5. Changes in content of cross-linking glycans throughout blueberry fruit ripening. Ripening stages are defined as in the caption of **Figure 1**. Two independent extractions were done for each developmental stage, and samples were measured in triplicate. The standard deviations are shown.

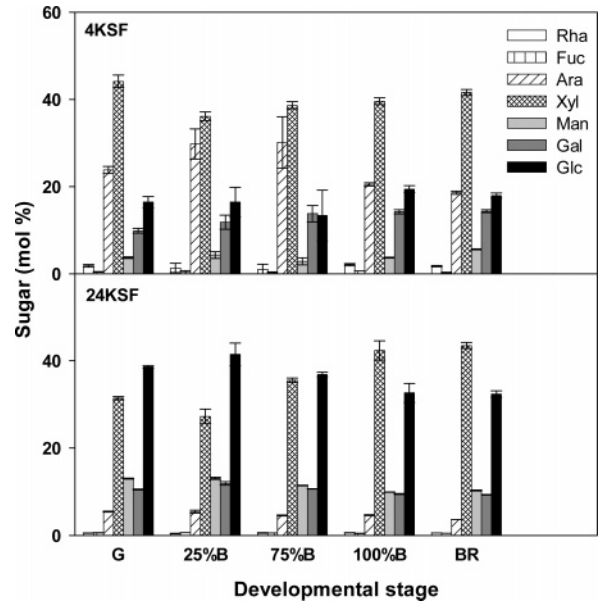

Figure 6. Neutral sugar composition (mole percent) of blueberry 4KSF and 24KSF throughout development. Ripening stages are defined as in the caption of **Figure 1**. Two independent extractions were done for each developmental stage, and samples were measured in duplicate. The standard deviations are shown.

a decrease in xyloglucan content increases the relative xylan content, that is, the relative content of Xyl. Short sclereids have been reported to be present in blueberries (*20*). Sclereids, so-called stone cells, are dead cells with thick secondary cell walls. Secondary cell walls are often rich in xylan polymers, so this could contribute to the high Xyl content observed in blueberry. Further study involving characterization of the glycosidic linkage content of this hemicellulose fraction would be required to provide a more certain explanation of the ripening-associated changes that are occurring.

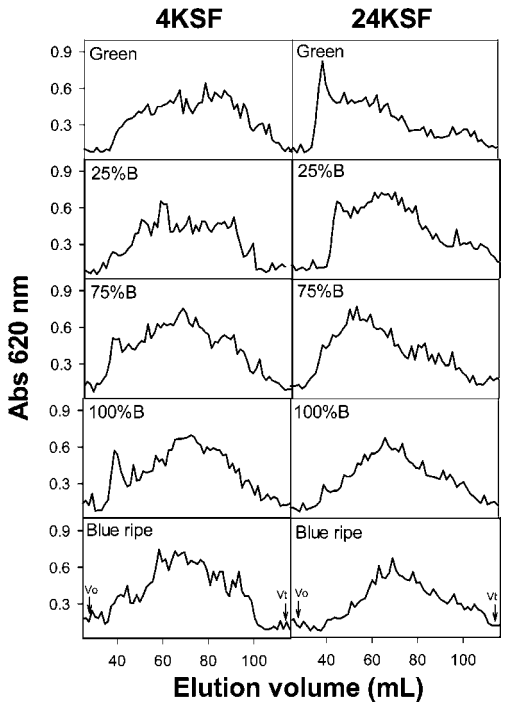

Figure 7. Size exclusion chromatography profiles of 4KSF and 24KSF from five blueberry fruit ripening stages fractionated on Sepharose CL-4B. Column fractions (1.5 mL) were assayed for neutral sugar content using the anthrone method (*9*). Vo, void volume; Vt, total volume.

The loosely bound glycans, particularly arabinoxylans, of the 4KSF showed no changes in molecular weight profile during ripening (**Figure 7**). A similar absence of depolymerization has been reported in comparable fractions from many other fruits for which downshifts in hemicellulosic polymer size are limited to polymers tightly bound to cellulose (*21*), that is, polymers extracted only by stronger base. In contrast, a clear downshift in molecular size was observed in the blueberry fruit 24KSF throughout development (**Figure 7**). From the 25%B to the 100%B stage the peak eluting at 45 mL almost disappeared; later in blueberry development the reduction in polymer size continued but to a lesser extent. Depolymerization of matrix glycans is thought to be an important contributor to fruit softening (*6*) and, with the exception of raspberry (*7*), a reduction in molecular size of these components has been observed in all species tested (*6*). Depolymerization of cross-linking glycans is usually accompanied by a reduction in pectin polymer size. The results observed for blueberry cv. Duke in which the fruit soften without changes in pectin size at any point in ripening are more similar to what has been found in pepper, apple, banana (*6*), and some strawberry varieties (*15*). This result suggests that the cell wall metabolism contribution to ripening-associated softening in blueberry is accomplished mainly by modification of the hemicelluloses. The possibility of pectin matrix affecting softening in overripe stages should not be ruled out, however. This has been shown in tomatoes in which pectin depolymerization takes place extremely late in ripening (*21*).

Model for Cell Wall Changes during Blueberry Development. Because blueberries ripen very little after harvest, they must be picked at full maturity to attain optimal flavor, and early picking is not a feasible strategy to obtain firmer fruit for retail. Mechanical harvesting also requires firmer varieties. A 20−30% reduction in fruit firmness was caused by machine harvesting (*22*). In addition, a 15% loss of firmness was observed during grading and sorting (*22*). The results reported

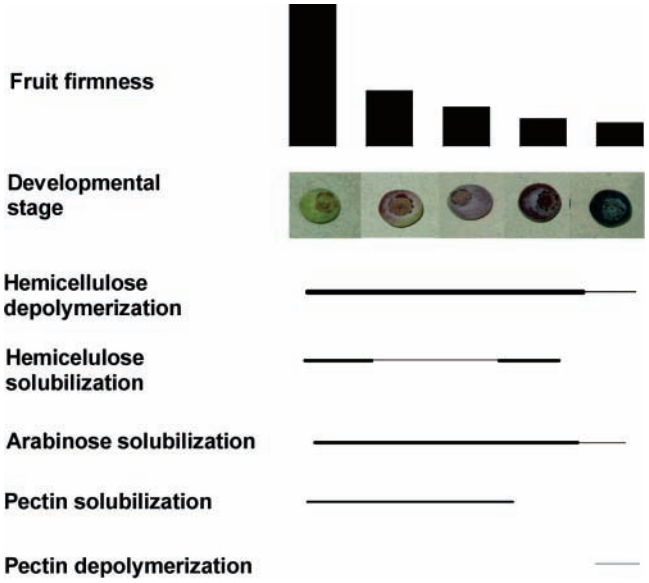

Figure 8. Proposed model for cell wall changes accompanying blueberry fruit ripening and softening.

here provide some fundamental information for understanding the biochemical basis of cell wall disassembly in blueberry and might be useful for guiding further work aimed at controlling softening. The unusual aspects of blueberry cell wall metabolism are as follows:

(1) The changes associated with blueberry fruit cell wall disassembly are quite different from those reported for most berries studied to date with a few similarities to some but not all strawberry varieties. The main cell wall modification observed was solubilization of pectin at an early ripening stage (**Figure 8**). As ripening progresses, substantial Ara solubilization is detected in both pectins and hemicelluloses; however, only the hemicellulosic polymers also undergo depolymerization.

(2) Xyl is the most abundant sugar in the blueberry fruit cell wall, and this is different from what has been reported for other fruits. Further studies to characterize the Xyl-rich polysaccharides might be valuable. A recent health-oriented nutritional practice is to encourage the consumption of food ingredients known as prebiotics, "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon, and thus improve host health" (*23*). Plant cell walls are known to be a potential source of pharmacologically active polysaccharides (*24*). Several oligosaccharides have been reported to possess prebiotic activity (*25*). Recent work has shown that dietary supplementation with xylooligosaccharides may have beneficial outcomes (*26*). Xylooligosaccharides may favor the selective growth of *Bifidobacterium* spp. (*27*, *28*), which provide benefits by suppressing the production of short-chain fatty acids by entero-putrefactive and pathogenic intestinal bacteria and, thus, facilitating nutrient absorption. This possibility has generated an increased commercial interest in these nondigestible oligosaccharides. The xylan-type polysaccharides are known to occur in several structural varieties in terrestrial plants (*24*). The richest sources of xylans are represented in many nonedible tissues, including graminaceous monocots (grasses) and woody tissues of dicots. In grasses, the branched arabinoxylans and mixed-linkage β-glucans are hypothesized to serve the role that xyloglucans play in dicotyledons (*29*). Because blueberry cell walls are rich in Xyl, presumably representing xylans, it would be useful to test whether or not some of the beneficial effects of consumption of the fruit also are due to prebiotic action.

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