Olive Fruit Cell Wall: Degradation of Cellulosic and Hemicellulosic Polysaccharides during Ripening

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Cellulose and hemicelluloses obtained from the cell walls of partially depectinated olives have been studied at three stages of ripening (green, cherry, and black). Hemicelluloses were fractionated into two groups, the amounts of which diminished during ripening: those soluble in 4% KOH diminished between the cherry and black stages, whereas those soluble in 24% KOH did so between the green and cherry stages. Arabinoxylans, xyloglucans, and homo- and/or rhamnogalacturonans to a lesser extent were present in these fractions. After ion exchange and size exclusion chromatographies, decreases in the molecular weights of hemicelluloses, mainly in the neutral fractions, were observed. The amount of cellulose also decreased, but at the second stage of the ripening process. Approximately 2 mg/fruit of glucose was lost from cellulose, and the amount of uronic acids increased (0.23 mg/fruit).

Keywords: *Cell wall; cellulose; hemicellulose; olives; ripening*

INTRODUCTION

Modifications of cell wall polysaccharides are the main factors responsible for the softening of vegetables and fruits during ripening and processing. Pectins, hemicelluloses, and cellulose are the three groups of polysaccharides implicated in these processes. Initially, research focused on the pectins; however, cellulose and especially hemicelluloses were later considered to have important roles in softening processes. In this respect, studies on changes in hemicelluloses during ripening have been recently performed on several fruits, including the peach (*1*, *2*), avocado (*3*), mango (*4*), strawberry (*5*), apple (*6*), and melon (*7*).

The study of olive fruit ripening is of great interest in Spain because the ripening stage of the fruit influences the processing and industrial production of olive oil as well as the production of pickled olives. In this paper, research has focused on hemicellulose and cellulose modifications during ripening, in addition to establishing the relationship between polysaccharide changes that take place in the cell wall of the olive with that of enzyme activity responsible for cell wall degradation reported in previous papers (*8*−*16*).

MATERIALS AND METHODS

Samples. Olive samples of Hojiblanca variety at three stages of ripeness [green (G), cherry (C), and black (B)] were provided by Agrosevilla-Aceitunas (La Roda de Andalucía, Sevilla, Spain). Fresh olives were frozen at −20 °C until required for the isolation of cell wall material (CWM).

Preparation of Partially Depectinated CWM. CWM was prepared according to previously reported methods (*17*, *18*) and afterward partially depectinated following extraction with 0.5 M imidazole (Fluka, puriss., Buchs, Swizerland)−HCl (Panreac, puriss., Barcelona, Spain) (pH 7) and 50 mM sodium carbonate (Sigma, Barcelona, Spain), as previously described (*19*).

Extraction of Hemicellulosic Polysaccharides. Partially depectinated CWM (from 1 g of original CWM) was treated as follows: (i) 4% potassium hydroxide (Merck, for analysis, Darmstadt, Germany), 20 mM in sodium borohydride (Sigma) (two times, 100 mL) for 12 h at 4 °C for the first extraction and 1 h at room temperature for the second extraction (K1SF); (ii) 24% potassium hydroxide, 20 mM in sodium borohydride (two times, 100 mL) at room temperature for 12 h for the first extraction and 1 h for the second extraction (K2SF); (iii) solid residue (α-cellulose) washed until its filtrate was neutral and then lyophilyzed (CEL). Each liquid extract (K1SF and K2SF) was neutralized with glacial acetic acid (Merck, for analysis), dialyzed against water, and lyophilized. Solid residues were then redissolved in the initial buffer of ion exchange chromatography.

Polysaccharide Analysis. Glycosyl compositions of the different fractions were determined after hydrolysis with 2 N trifluoroacetic acid (Sigma) (121 °C, 1 h) (*20*) and quantified as alditol acetates (*21*).

Cellulosic residue was hydrolyzed as follows: 2-h hydrolysis with 72% H_2SO_4 (Panreac, for analysis) at 40 °C, dilution to 6% H_2SO_4, and 3-h hydrolysis at 100 °C. The hydrolysate was filtered through glass wool. An aliquot containing ∼100 μg of sugars was taken (based on anthrone assay). Inositol (Sigma) (25 μg) was added as the internal standard, and the mixture was neutralized with 2 N NH_4OH (Panreac). Neutral sugars (NS) were quantified in the neutralized sample by reduction, acetylation, and gas chromatography (GC) measurements.

Uronic acids (UA) in the different fractions were quantified by the phenyl−phenol method (*22*).

Ion Exchange Chromatography. Acidic polysaccharides were separated from neutral polysaccharides in K1SF and K2SF by fractionation on 5-mL Hi-Trap columns (anionic exchanger: Q-Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in 0.01 M imidazole−HCl buffer, pH 7. The neutral fraction was recovered by washing the column with the same buffer, whereas the acidic fractions were obtained by stepwise increases in concentration of the running buffer: 0.5, 1.0, 1.5, and 2.0 M.

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Table 1. Glycosyl Composition of the 4% KOH-Soluble Fraction of Olive Fruit at Three Stages of Ripening Prior to Ion Exchange Chromatography

	Glycosyl Composition of 4% KOH-Soluble Fraction[a]							
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
G	0.05 ± 0.01 a	nd	0.46 ± 0.02 a	2.49 ± 0.21 ab	0.10 ± 0.00 a	0.17 ± 0.01 a	0.42 ± 0.14 a	0.59 ± 0.04 a
C	0.06 ± 0.00 a	nd	0.42 ± 0.03 a	2.96 ± 0.23 a	0.08 ± 0.00 a	0.15 ± 0.02 a	0.41 ± 0.00 a	0.51 ± 0.07 ab
B	0.09 ± 0.01 b	nd	0.45 ± 0.05 a	1.81 ± 0.03 b	0.10 ± 0.01 a	0.18 ± 0.02 a	0.48 ± 0.02 b	0.43 ± 0.02 b

	Glycosyl Composition of 4% KOH-Soluble and Insoluble Fractions[b]								
		glycosyl composition (%)							
	%[c]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
soluble fraction									
G	38	1	0	13	52	5	5	20	4
C	37	2	0	10	53	5	5	17	8
B	52	2	0	11	44	5	7	27	4
insoluble fraction									
G	62	1	0	9	73	0	1	1	14
C	63	1	0	7	79	0	1	1	10
B	48	2	0	15	69	0	2	1	9

[a] Results are expressed a milligrams per fruit and are the average value of four replicates. Different letters in the same column indicate significant differences. [b] Results are expressed as percent. [c] This percentage represents the percentage of the fraction on the total sugar recovered.

Fractions were assayed for NS (*23*) by using the anthrone method. Those corresponding to peaks were concentrated in a rotary evaporator at 30 °C, dialyzed against distilled water (MW cutoff = 12000 Da), and freeze-dried.

High-Performance Size Exclusion Chromagraphy (HPSEC) Analysis. The molecular weight distribution of the fractions was determined on a Hewlett-Packard 1100 series system, fitted with two different Tosohaas columns (7.8 mm i.d. × 30 cm) in sequence: TSK gel GMPWXL and TSK gel G3000PWXL, after calibration with 250, 110, 40, and 6 kDa dextrans (Fluka). The running buffer was 0.1 M imidazole−HCl, pH7, and the samples were eluted at a flow rate of 0.6 mL/min. The injection volume was 20 μL, and the fractions collected were each of 0.3 mL.

Statistical Analysis. The data were statistically analyzed through their variance. Means were compared by Duncan's multiple-range test ($P < 0.05$).

RESULTS AND DISCUSSION

Cell wall hemicelluloses and α-cellulose were isolated. Hemicelluloses (K1SF and K2SF) were studied by GC, ion exchange chromatography, and HPSEC. The glycosyl composition of CEL was also studied. Modifications that took place in these fractions will be discussed and an overall mechanism for the olive fruit ripening process presented.

Composition of Hemicellulosic Cell Wall Fractions. The amounts of NS and UA in hemicelluloses and cellulose are presented in Figure 1. In K1SF, significant decreases occurred between C and B in both UA and NS. In K2SF, the decrease in UA was significant throughout the whole process, whereas NS decreased only between G and C. In addition to these findings, it is interesting to note the considerable decrease in CEL fraction between C and B.

4% KOH-Soluble Fraction. In the general composition (Table 1), there were significant differences between G and B in UA and between C and B in NS. Xylose decreased by >1 mg/fruit, whereas glucose increased significantly (0.07 mg/fruit). When this fraction was redissolved in running buffer (0.01 M imidazole−HCl, pH 7) prior to ion exchange chromatography, an insoluble residue was obtained. The compositions of both soluble and insoluble fractions are also presented in Table 1. The insoluble fraction consisted mainly of acidic arabinoxylans and was the major fraction. The xylose

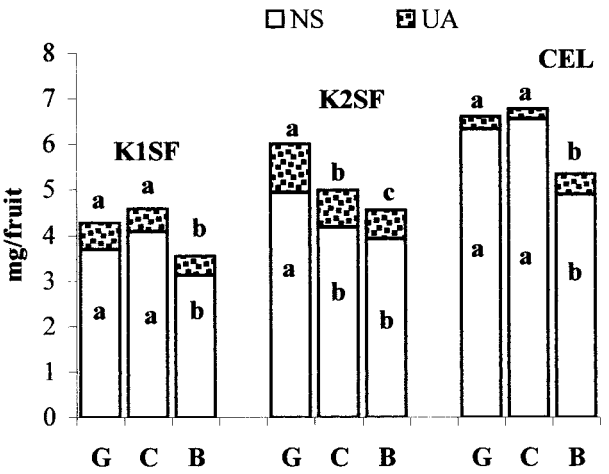

Figure 1. Composition of K1SF, K2SF, and CEL of olive fuit at three stages of ripening (G, C, and B). NS and UA are expressed as milligrams per fruit. Different letters indicate significant differences.

lost between C and B was released from this fraction (its percentage decreased from 62 to 48%), and the percentage of xylose in the total fraction also decreased by 1.15 mg/fruit. The presence of xylans in edible fruits is very restricted because these polysaccharides are related to secondary growth in the cell wall. The presence of stone cells has been reported in olive fruits (*24*−*26*) and pears (*27*), with the composition of these fruits having a significant percentage of xylans. In the soluble fraction (Table 1), the presence of xyloglucans could be suggested in addition to that of arabinoxylans. The percentage of xylose also decreased in this fraction, supporting the hypothesis that a loss of xylans takes place during ripening. The high proportion of arabinose could indicate the presence of pectic polymers, in addition to that of arabinoxylans.

Four peaks were isolated using ion exchange chromatography (Table 2), consisting of the neutral peak and three acidic peaks (A1, A2, and A3) eluting with 0.5, 1.0, and 1.5 M imidazole buffer, respectively. The quantitatively most important was the neutral peak (>50%). As a result of ripening, the neutral polysaccharides increased and the acidic diminished.

Table 2. Glycosyl Composition of the Four Fractions Eluted by Ion Exchange Chromatography (Neutral, A1, A2, and A3) from the 4% KOH-Soluble Fraction

		glycosyl composition (%)							
	%[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
neutral									
G	53.75	1	1	8	31	8	11	40	0
C	55.67	1	1	8	33	6	11	41	0
B	67.33	1	1	10	37	6	9	37	0
A1									
G	21.25	1	0	6	49	2	4	13	24
C	17.53	2	1	9	40	1	3	8	37
B	17.82	2	0	6	55	2	4	10	20
A2									
G	8.75	6	0	32	32	2	8	14	3
C	6.49	2	0	7	67	1	4	8	11
B	6.93	5	1	12	42	2	4	17	18
A3									
G	16.25	7	0	28	10	2	7	16	30
C	10.31	3	0	15	27	1	3	11	39
B	7.92	5	0	16	25	1	4	10	38

[a] This percentage represents the percentage of the fraction on the total sugar recovered after chromatography.

The composition of the neutral fraction suggested mainly the presence of xyloglucans. There was a small proportion of arabinose that could indicate the presence of lesser amounts of arabinoxylans. The Xyl/Glc ratio was 0.78 in G, 0.82 in C, and 0.99 in B. As arabinose also increased, it could be suggested that the proportion of arabinoxylans increased with ripening. These arabinoxylans could arise from the acidic fractions that decreased during ripening due to the loss of uronic acids.

Acidic fractions seemed to be composed mainly of acidic arabinoxylans, although the presence of rhamnogalacturonans could also be suggested because of the higher percentage of rhamnose and UA. In the A3 fraction, the percentage of uronic acids was considerable (30−40%). Together with the lower proportion of xylose, these data could indicate the presence of rhamnogalacturonans together with homogalacturonans. UA percentages increased, particularly in A2 and A3.

Only the neutral fraction was studied by HPSEC (Figure 2). The profiles show a broadening of the eluted peak and a shift of the absorbance maximum to lower molecular weights. The range of molecular weight varied between 250 and 40 kDa, but the absorbance maximum was between 250 and 110 kDa and nearer to that of the second standard as ripening progressed. Slight diminutions in molecular weight of the loosely bound hemicelluloses have been described in peaches (*2*) and persimmon (*Diospyros kaki* L.) (*28*), although in the latter case an increase in apparent molecular weight prior to the decrease was reported.

24% KOH-Soluble Fraction. Arabinose was the major sugar in the three samples analyzed (Table 3), with xylose, glucose, and UA detected in appreciable amounts. Glucose decreased significantly only between G and C, arabinose between G and B, and xylose between C and B. This diminution in hemicelluloses has also been described in mango fruit (*4*), persimmon (*28*), and avocado (*3*). In the case of strawberries (*5*), it is interesting to note that a diminution of arabinose was also quantified in this fraction, although it was not the major sugar. In this fraction, as in K1SF, a soluble fraction and an insoluble fraction were isolated (Table 3). Unlike K1SF, however, the soluble fraction was the major one and underwent an important diminution in arabinose, the main sugar in its composition. As for the

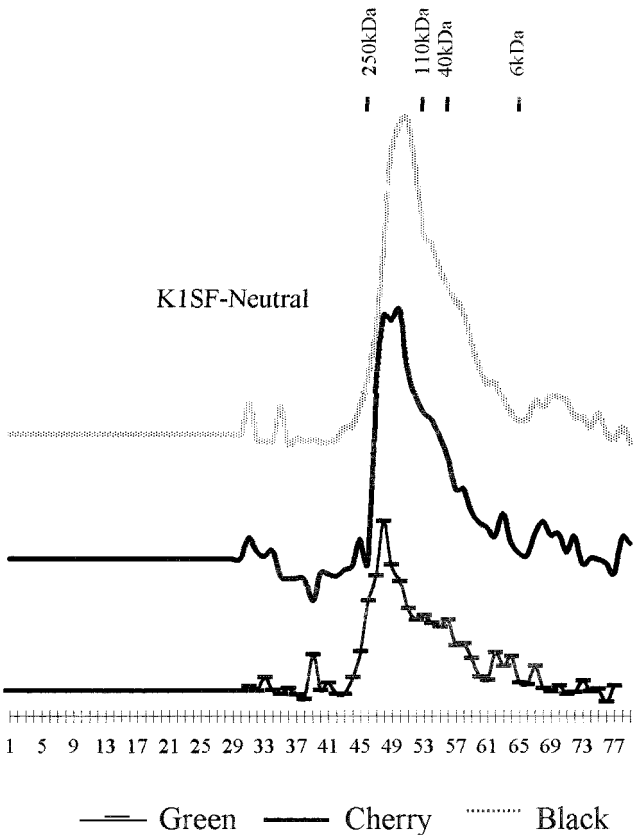

Figure 2. Profiles of elution of neutral fraction from K1SF of olive fruit at three stages of ripening (G, C, and B). Abscissa: absorbance at 630 nm (anthrone assay).

results from other fruits (*2*, *28*), increases in the concentration of potasium hydroxide at the extraction step increased the proportion of xyloglucan-like polymers. However, xylose was the major sugar in the insoluble material, this fraction not showing variations during ripening.

After ion exchange, four fractions eluted from the soluble polysaccharides (Table 4): neutral and three acidic (A1, A2, and A3) eluting with 0.5, 1.0, and 1.5 M imidazole buffer, with the major fractions being the neutral and A3 fractions.

The percentage of the neutral fraction increased with ripening and seemed to be composed mainly of xyloglucans. The percentages of arabinose in G and C were very similar to those of the neutral polysaccharides of the previous fraction (K1SF), also suggesting the presence of arabinoxylans in this case. However, the percentage of arabinose in B increased to ∼20%, which should indicate that the amount of arabinans was elevated in this fraction.

The next fraction in quantity was A3, which had a typically pectic composition, the UA percentage increasing with ripening (pectic side chains, mainly arabinans, could elute in the neutral fraction as ripening progressed). The presence of rhamnogalacturonans in K2SF in amounts of such magnitude (>30%) gives an idea of the strong relations that exist between xyloglucans, arabinoxylans, rhamnogalacturonans, and cellulose. This higher proportion of pectins associated with the more tightly bound hemicellulose extract from the three stages of ripening has also been reported for other fruits such as peaches (*1*, *2*), persimmons (*28*), and pears (*27*). A2 had a composition very similar to that of A3 but

Table 3. Glycosyl Composition of the 24% KOH-Soluble Fraction of Olive Fruit at Three Stages of Ripening Prior to Ion Exchange Chromatography

	Glycosyl Composition of 24% KOH-Soluble Fraction[a]							
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
G	0.16 ± 0.02 a	nd	1.90 ± 0.36 a	1.05 ± 0.04 a	0.38 ± 0.02 a	0.42 ± 0.08 a	1.02 ± 0.03 a	1.08 ± 0.12 a
C	0.13 ± 0.02 a	nd	1.51 ± 0.38 ab	0.98 ± 0.04 a	0.34 ± 0.05 a	0.36 ± 0.07 a	0.85 ± 0.01 b	0.82 ± 0.02 b
B	0.14 ± 0.01 a	nd	1.27 ± 0.06 b	0.89 ± 0.01 b	0.41 ± 0.01 a	0.38 ± 0.01 a	0.83 ± 0.04 b	0.64 ± 0.05 c

	Glycosyl Composition of 24% KOH-Soluble and Insoluble Fractions								
		glycosyl composition (%)							
	%[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
soluble fraction									
G	81.00	3	0	40	11	7	7	25	14
C	89.33	2	0	37	10	8	8	23	12
B	82.77	3	0	20	6	11	10	23	8
insoluble fraction									
G	19.00	1	0	10	65	2	3	6	12
C	10.67	0	0	10	66	2	4	6	12
B	17.22	3	0	14	60	3	3	8	12

[a] Results are expressed as milligrams per fruit and are the average value of four replicates. Different letters in the same column indicate significant differences. [b] This percentage represents the percentage of the fraction on the total sugar recovered.

Table 4. Glycosyl Composition of the Four Fractions Eluted by Ion Exchange Chromatography (Neutral, A1, A2, and A3) from the 24% KOH-Soluble Fraction

		glycosyl composition (%)							
	%[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
neutral									
G	43.38	0	1	7	12	19	13	48	0
C	50.61	0	0	7	14	20	12	46	0
B	53.32	1	1	21	18	17	12	30	0
A1									
G	5.94	2	0	42	25	2	5	10	13
C	7.48	1	0	35	26	2	4	12	18
B									
A2									
G	13.7	6	0	51	6	1	6	6	23
C	9.93	5	0	61	4	0	9	6	14
B	8.54	5	0	38	10	1	6	10	29
A3									
G	36.99	6	0	36	2	0	6	5	44
C	31.97	2	0	28	4	0	3	4	59
B	34.15	4	0	29	2	0	4	4	57

[a] This percentage represents the percentage of the fraction on the total sugar recovered after chromatography.

poorer in UA, although its percentage in the total fraction was lower. The A1 fraction clearly was composed of arabinans and arabinoxylans.

As shown in Figure 3, there was a change in the elution profile of the neutral fraction. For green olives, a broad peak without a marked maximum in absorbance was observed. As ripening progressed, the profile became sharper, with the maximum being shifted toward lower molecular weights. For black olives, this maximum occurred in a molecular weight range between 110 and 40 kDa. The decrease in molecular weight was more pronounced between the green and cherry stages as has been described for strawberries (*5*). Changes in the A3 fractions were less apparent. In green fruits, there was a sharp peak slightly below 250 kDa, and a shoulder between 110 and 40 kDa. The peak broadened in cherry fruits, whereas in black fruits it appeared as two different peaks, suggesting a decrease in the molecular weight of this pectic fraction. This difference between the molecular weight ranges of the neutral and acidic fractions found in olives, in which the latter displays higher molecular weights, has also been described for peaches (*2*). In this case, the acidic peak was almost unchanged during ripening, thus increasing the proportion of medium and low molecular weight species in the neutral peak.

α-Cellulose Fraction. The glycosyl composition of α-cellulose is presented in Table 5. A diminution of 2 mg/fruit was measured between C and B, which was caused by a significant loss of glucose, as in the case of cherry fruits (*Prunus avium* L. Bigarreau Burlat) (*29*). The other sugars were present in amounts of <5%, although in B fruits those percentages increased due to the decrease of glucose.

The decrease in CEL is not always apparent in fruit ripening. In Spanish pear (*Pyrus communis* cv. Blanquilla) (*27*) and mango (*Mangifera indica* L.) (*4*) there were small increases, with minor diminution in avocado (*Persea americana* Mill) (*3*) or no changes reported in peach (*Prunus persica*) (*1*) and tomato (*Lycopersicum esculentum* L.) (*30*, *31*).

Overall Mechanism of Olive Fruit Ripening. A series of enzymatic activities have been described during the growth and ripening of olive fruits. At the green ripening stage, the activities of several cell wall-linked glycosidases have been measured: α-mannosidase, α-galactosidase, β-glucanase, α-arabinosidase, β-galactosidase, and β-xylosidase (*11*). In the same paper, some endo-activity was also reported arising from carboxymethylcellulase (CMCase), xyloglucanase (XGase), and endopolygalacturonase (endoPG). Later in the ripening process but still at the green stage, the activity of cytoplasmic glycosidases (*14*, *15*) has been described, with activities similar to those cited for cell wall-linked glycosidases, in addition to pectinesterase activity (*16*). At the cherry stage, all of the cell wall-linked and cytoplasmic enzymes reach their maximum activities. At that point, cytoplasmic endoglucanases and endoPG (*8*−*10*, *12*, *13*) are present in the fruit at very low levels, reaching their maximum activity at the black overripe stage. At the black stage, all of the glycosidases undergo a decrease in their activity, with only the wall-linked and cytoplasmic endoPG and endoglucanases continuing to increase.

In this work and in a previous paper (*32*), the cell wall changes that take place during olive ripening have been discussed. Between green and cherry fruits, the most important changes that take place are the solubilization

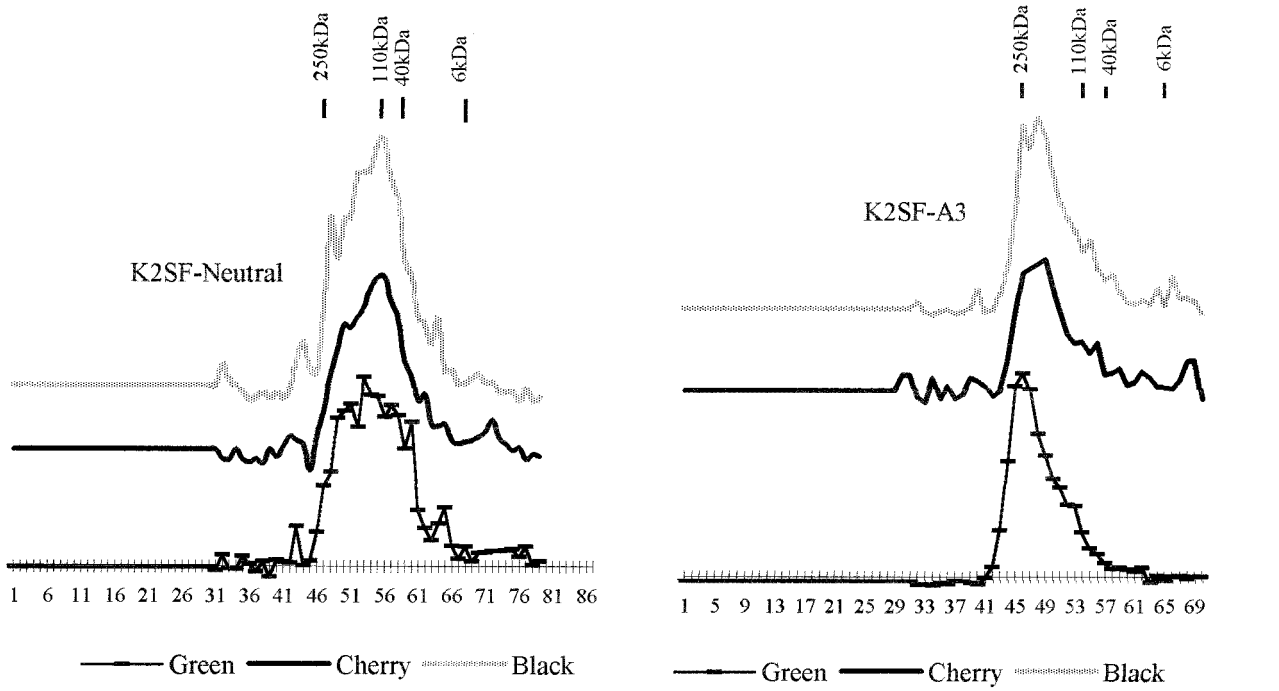

Figure 3. Profiles of elution of neutral and A3 fractions from K2SF of olive fruit at three stages of ripening (G, C, and B). Abscissa: absorbance at 630 nm (anthrone assay).

Table 5. Glycosyl Composition of the Cellulose Fraction of Olive Fruit at Three Stages of Ripening (G, C, and B)[a]

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
G	nd	nd	0.10 ± 0.01 a	0.06 ± 0.01 a	0.01 ± 0.01 a	0.02 ± 0.01 a	6.14 ± 0.03 a	0.28 ± 0.12 a
C	nd	nd	0.20 ± 0.01 a	0.06 ± 0.01 a	nd a	0.05 ± 0.01 a	6.24 ± 0.04 a	0.22 ± 0.02 a
B	nd	nd	0.20 ± 0.01 a	0.08 ± 0.01 a	0.01 ± 0.01 a	0.09 ± 0.01 a	4.44 ± 0.43 b	0.45 ± 0.05 b

[a] Different letters in the same column indicate significant differences.

of homogalacturonans and decreases in the yield of tightly bound hemicelluloses and in the molecular weight of their neutral fractions. In addition to these changes, a slight decrease in molecular weight of neutral hemicelluloses loosely bound to the cell wall has also been described here. The enzymes that could produce these modifications in olive fruit are linked to the cell wall structure. Although the activities of endoPG and endoglucanases (CMCase and XGase) are very low compared with those of the soluble enzymes (*11*, *15*), their accessibility to the specific substrates must be higher, leading both to the release of homogalacturonan fragments and to the molecular weight reduction in neutral hemicelluloses, which are mainly composed of xyloglucans. The hydrolysis of xyloglucans may be the reason for the increase of glucose in the phosphate-soluble fraction (PSF) (*32*). Given that the presence of a mixed glucan in the olive cell wall has been suggested in previous papers (*18*, *25*), such degradation could also increase glucose in the PSF.

Between cherry and black fruits, the more significant modifications are those that take place to hemicelluloses and cellulose. The yield of loosely bound hemicelluloses decreases, the molecular weight of neutral hemicelluloses continues to decrease, and the cellulose content falls. Minor changes occur in pectic fractions, with rhamnogalacturonan side chains being released in addition to the loss of homogalacturonan fragments. The high endoglucanase activity found at this stage of ripening, both cell wall-linked and cytoplasmic, is implied in the degradation of cellulose and hemicelluloses. Prior glycosidase activity could have facilitated the action of endoenzymes, activities that lead to the release of rhamnogalacturonan side chains in the case of pectins.

In addition to these degradative processes, the synthesis of polysaccharides during ripening, mainly the hemicelluloses, must take place (*32*). A study of the enzymes implicated in this process would provide valuable information on this subject. With such knowledge, a complete mechanism of cell wall polysaccharide turnover during olive fruit ripening could be postulated.

ABBREVIATIONS USED

B, black stage; C, cherry stage; CEL, cellulose; CWM, cell wall material; CMCase, carboxymethylcellulase; G, green stage; K1SF, 4% KOH-soluble fraction; K2SF, 24% KOH-soluble fraction; NS, neutral sugars; PG, polygalacturonase; PSF, phosphate-soluble fraction; UA, uronic acids; XGase, xyloglucanase.

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