Changes in Oxygen-Scavenging Systems and Membrane Lipid Peroxidation during Maturation and Ripening in Blackberry

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Maturation and ripening of blackberry (*Rubus* sp.) fruit was accompanied by decreased activities of oxygen-scavenging enzymes [superoxide dismutase (EC 1.15.1.1), glutathione-peroxidase (EC 1.11.1.9), catalase (EC 1.11.1.6)] and enzymes in the ascorbate−glutathione cycle [ascorbate peroxidase (EC 1.11.1.11), monodehydroascorbate reductase (EC 1.6.5.4), dehydroascorbate reductase (EC 1.8.5.1), and glutathione reductase (EC 1.6.4.2)]. Nonenzyme components in the ascorbate−glutathione cycle such as ascorbate (AsA), dehydroascorbate (DHAsA), glutathione (GSH), and oxidized glutathione (GSSG) and the ratios of AsA/DHAsA, GSH/GSSG were also decreased. These decreases in antioxidant capacity were correlated with increases in the ratios of saturated to unsaturated fatty acid of polar lipids and free sterols to phospholipids, thus contributing to decreased fluidity, enhanced lipid peroxidation, and membrane deterioration, which may be associated with ripening and senescence in blackberry fruit.

Keywords: *Antioxidants; blackberry; lipid peroxidation; oxygen-scavenging enzymes; maturation and ripening*

INTRODUCTION

Fruit maturation and ripening are accompanied by alterations in the physical properties of the lipid matrix in membranes and changes in the activity of membrane-bound enzymes (*1*, *2*). Increases in ion leakage, decreases in the fluidity of plasma membranes, losses of membrane integrity, and accumulation of peroxidized lipids in membranes occur during maturation and ripening of muskmelon (*3*), apple (*1*), and tomato fruit (*4*).

Free radical-induced oxidative stress plays a role in fruit maturation, ripening, and senescence (*5*, *6*). Increased levels of H_2O_2 and lipid peroxides have been detected in ripening fruits (*7*). Lacan and Baccou (*8*) found that senescence in nonnetted muskmelon fruit is the result of lipid peroxidation by free radicals, membrane phospholipid breakdown, and a drop in the level of antioxidants. High levels of superoxide dismutase (SOD) and catalase (CAT) are involved in delaying the senescence process (*8*). In tomato fruit, SOD and peroxide activities declined steadily from the immature green stage to the red-ripe stage (*9*). Rogiers et al. (6) showed that oxidative stress increased progressively during ripening, probably as a consequence of reduced activities of key enzymes (SOD and CAT) responsible for quenching active oxygen species. However, little information is available on changes in free radical-scavenging systems associated with the breakdown of membrane lipids during maturation and ripening in berry crops. This study was undertaken to determine the changes in antioxidant systems, enzymes in the ascorbate−glutathione cycle, and the role of these antioxidant systems in the degradation of membranes during maturation, ripening, and senescence of blackberries.

MATERIALS AND METHODS

Chemicals. *N*-Ethylmaleimide and trichloroacetic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ethylenediaminetetracetic acid, disodium salt (EDTA, dihydrate-Na_2 EDTA·$2H_2O$), was obtained from Life Technologies (Rockville, MD). Ascorbate oxidase, bathophenanthroline, dicoumarol, digitonin, dithiothreitol (DTT), glutathione (oxidized form), glutathione (GSH, reduced form), glutathione reductase (GR), guaiacol, hydrogen peroxide (30% w/w), methionine, β-nicotinamide adenine dinucleotide (β-NADH, reduced form), β-nicotinamide adenine dinucleotide phosphate (β-NADPH, reduced form), nitro blue tetrazolium (NBT), phenylmethanesulfonyl fluoride, polyvinylpolypyrrolidone, and riboflavin (vitamin B_2) were purchased from Sigma Chemical Co. (St. Louis, MO). Heptadecanoate, *n*-heptadecanoic acid, lathosterol (cholest-7-en-3β-ol), palmitate, stearate, oleate, linoleate, linolenate, β-sitosterol, cholesterol, and campesterol were obtained from Supelco, Inc. (Bellefonte, PA).

Fruit Sample Preparation. Thornless blackberries (cv. Black Satin, Chester Thornless, Smoothstem, and Thornfree) used in this study were grown at the Henry A. Wallace—Beltsville Agricultural Research Center. Collections of berries were carried out over a period of 2 weeks during the fruit maturation season. Samples of blackberry fruits were harvested from six to eight bushes of each cultivar at various maturity stages. On the basis of fruit surface color, maturity was classified as green, pink, commercially ripe, and over-ripe. Undamaged berries were selected, randomized, frozen in liquid nitrogen, and then stored at −80 °C until they were assayed for enzyme and membrane lipid extraction.

Catalase (CAT, EC 1.11.1.6), Glutathione Peroxidase (GSH-POD, EC 1.11.1.9), and GR (EC 1.6.4.2). Triplicate fruit tissue [10 g of fresh weight (fwt)] was homogenized in 5 mL of 0.1 M Tris-HCl buffer (pH 7.8) containing 2 mM EDTA-

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Na and 2 mM DTT. The homogenate was centrifuged at 20000*g* for 30 min at 4 °C, and the supernatant was used for the CAT, GSH-POD, and GR assays.

CAT activity was determined by the floating disk method (*10*). The disks were excised with a 6-mm-diameter cork borer from Whatman 3 mm chromatographic paper. Ten microliters of crude enzyme extract was applied to each disk, and the disks were placed in a vial containing 5 mL of 30 mM H_2O_2 at 25 °C. The elapsed time for the disks to float from the bottom to the top of the solution was determined with a stopwatch. Ten to 20 replicates of individual disks were used for each crude extract. The activity of CAT in the blackberry extract was calculated according to the activity of bovine liver catalase (δ = 11000 units/mg of protein). One unit of CAT activity was equal to 1 μmol of H_2O_2 degraded per minute at 25 °C.

GSH-POD activity was determined using the method of Tappel (*11*) with a slight modification. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.0), 0.4 mM EDTA, 1.0 mM NaN_3, 1.0 mM H_2O_2, 1.0 mM GSH, 0.15 mM NADPH, 1 unit of GR, and 100 μL of enzyme extract. The total reaction volume was 1.0 mL. The reaction was started by adding H_2O_2. GSH-POD activity was determined by the rate of NADPH oxidation at 340 nm via a spectrophotometer (Shimadzu UV-160A, Shimadzu Scientific Instruments, Columbia, MD). Enzyme activity was expressed as nanomoles of NADPH oxidized per milligram of protein per minute.

GR activity was assayed according to the method of Smith et al. (*12*). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 0.5 mM oxidized glutathione (GSSG), 2 mM EDTA, 0.15 mM NADPH, and 0.1 mL of crude enzyme extract. The total reaction volume was 1.0 mL. The activity of GR was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm. The reaction was started by adding GSSG, and the rate of oxidation was calculated using the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}). GR activity was expressed as nanomoles of NADPH oxidized per milligram of protein per minute.

SOD (EC 1.15.1.1). Triplicate fruit tissue (10 g) was pulverized in a cold mortar and pestle with 10 mL of potassium phosphate buffer (0.1 M, pH 7.3) containing 1 mM EDTA and 2 mM DTT. The homogenate was strained through four layers of Miracloth and centrifuged at 12000*g* for 10 min at 4 °C. The supernatant was purified according to the method of Wang et al. (*13*) before the SOD enzyme activity was assayed.

Total SOD activity was assayed photochemically (*14*), on the basis of the photoreduction of NBT by light in the presence of riboflavin and methionine. NBT is reduced to blue diformazan, which has a strong absorbance at the 560 nm wavelength. Under aerobic assay conditions, SOD inhibits the formation of blue diformazan. NBT can be reduced by either pyridine nucleotide or $O_2^{\bullet -}$ (*15*). Dicoumarol was included in the reaction mixture to inhibit reduction by pyridine nucleotide (*15*) and to obtain a completely $O_2^{\bullet -}$-dependent reduction of NBT. The reaction mixture (1 mL) contained 100 μM dicoumarol, 1.3 μM riboflavin, 13 mM methionine, 0.05 M Na_2CO_3, 0.01 M sodium phosphate buffer (pH 7.8), and 0.1 mL of the enzyme extract; after 3 min, 63 μM NBT was added. The mixtures were illuminated by fluorescent lamp (light intensity was 170 μeinstein$\cdot m^{-2}\cdot s^{-1}$) for 3 min. Identical solutions held in the dark served as blanks. One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under assay conditions. Because inhibition is not linearly correlated with SOD concentration, a *V*/*v* transformation was used to obtain linearity (V = basic reaction rate without blackberry fruit extract and v = reaction rate with extract). Linear correlation gave the equation SOD units/mL = $(0.459V/v - 0.032) \times$ dilution factor. The correlation coefficient for this line was 0.985.

Ascorbate Peroxidase (AsA-POD, EC 1.11.1.11), Dehydroascorbate Reductase (DHAR, EC 1.8.5.1), Monodehydroascorbate Reductase (MDAR, EC 1.6.5.4), and Guaiacol Peroxidase (G-POD, EC 1.11.1.7). Triplicate fruit tissue (10 g) was pulverized in a cold mortar and pestle with 10 mL of potassium phosphate buffer (0.1 M, pH 7.3) containing 1 mM EDTA and 2 mM DTT. The homogenate was centrifuged at 12000*g* for 10 min at 4 °C. The supernatant was used for the AsA-POD, DHAR, MDAR, and G-POD assays.

AsA-POD activity was assayed according to the method of Amako et al. (*16*) by measuring the oxidation of ascorbate at 290 nm; the reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, 0.5 mM H_2O_2, and 0.1 mL of crude enzyme extract in a total volume of 1.0 mL. The reaction was started by adding H_2O_2. Enzyme activity was expressed as nanomoles of ascorbate oxidized per milligram of protein per minute.

DHAR activity was assayed by measuring the rate of NADPH oxidation at 340 nm (*17*). The reaction mixture contained 50 mM potassium phosphate (pH 6.1), 0.2 mM NADPH, 2.5 mM dehydroascorbate, 2.5 mM glutathione, 0.6 unit of GR (from spinach, EC 1.6.4.2), and 0.1 mL of crude enzyme. The reaction was started by adding dehydroascorbate. Enzyme activity was expressed as nanomoles of NADPH oxidized per milligram of protein per minute.

MDAR activity was assayed by measuring the rate of NADH oxidation at 340 nm (*18*). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.3), 0.2 mM NADH, 1.0 mM ascorbate, 1.0 unit of ascorbate oxidase, and 0.1 mL of crude enzyme extract in a total volume of 1.0 mL. The reaction was started by adding ascorbate oxidase (from *Cucurbita*, EC 1.10.3.3). Enzyme activity was expressed as nanomoles of NADPH oxidized per milligram of protein per minute.

The G-POD assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol as donor, 3 mM H_2O_2 as substrate, and 1.0 mL of crude enzyme extract. The total reaction volume was 3.0 mL. The rate of change in absorbance at 420 nm was measured, and the level of enzyme activity was expressed as the difference in absorbance (OD) per milligram of protein per minute.

Determination of Ascorbate (AsA) and Dehydroascorbate (DHAsA). AsA and DHAsA were determined using the methods of Arakawa et al. (*19*) and Nakagawara and Sagisaka (*18*). This assay is based on the reduction of ferric ion to ferrous ion with ascorbic acid in acid solution followed by formation of the red chelate between ferrous ion and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) that absorbs at 534 nm. Total ascorbate (ascorbate plus dehydroascorbate) was determined through a reduction of dehydroascorbate to ascorbate by DTT. Triplicate fruit tissue (10 g) was homogenized in 10 mL of cold 5% (w/v) trichloroacetic acid. The homogenate was filtered through four layers of Miracloth and centrifuged at 16000*g* for 10 min at 4 °C. The supernatant was used for the ascorbate and total ascorbate assay. The ascorbate assay mixture contained 0.1 mL of the sample extract, 0.5 mL of absolute ethanol, 0.6 M trichloroacetic acid, 3 mM bathophenanthroline, 8 mM H_3PO_4, and 0.17 mM $FeCl_3$. The final total volume was 1.5 mL, and the solution was allowed to stand at 30 °C for 90 min for the Fe^{2+}−bathophenanthroline complex to develop. The absorbance of the colored solution was read at 534 nm. The total ascorbate assay mixture contained 0.1 mL of the sample solution, 0.15 mL of 3.89 mM DTT, and 0.35 mL of absolute ethanol in a total volume of 0.6 mL. Then, the reaction mixture was left standing at room temperature for 10 min. After reduction of dehydroascorbate to ascorbate, 0.15 mL of 0.24% *N*-ethylmaleimide in ethanol and 0.15 mL of 20% trichloroacetic acid were added. The color was developed by adding the following reagents in this sequence: 0.15 mL of 0.4% (v/v) H_3PO_4−ethanol, 0.3 mL of 0.5% (w/v) bathophenanthroline−ethanol, and 0.15 mL of 0.03% (w/v) $FeCl_3$−ethanol. The final volume was 1.5 mL, and after incubation at 30 °C for 90 min, the absorbance at 534 nm was recorded. Dehydroascorbate concentrations were estimated from the difference of "total ascorbate" and "ascorbate" concentrations. A standard curve in the range 0−10 μmol of ascorbate or dehydroascorbate was used.

Measurement of Glutathione (GSH) and Oxidized Glutathione (GSSG). Triplicate fruit tissue (10 g) was homogenized in 5.0 mL of ice-cold 60.0 mM potassium phosphate solution with a chilled mortar and pestle under N_2 at 0 °C. The homogenate was filtered through four layers of Miracloth and the filtrate centrifuged at 20000*g* for 15 min at

Table 1. Activities of SOD, G-POD, GSH-POD, and CAT in Thornless Blackberry Fruit at Different Stages of Maturity[a]

cultivar	maturity	SOD (units/mg of protein)	G-POD (ΔA/mg of protein·min)	GSH-POD (nmol/mg of protein·min)	CAT (units/mg of protein)
Black Satin	green	13.8 ± 1.3	1.25 ± 0.06	34.2 ± 2.1	12.5 ± 0.6
	pink	12.0 ± 0.9	1.02 ± 0.01	30.1 ± 1.9	1.0 ± 0.1
	ripe	10.0 ± 0.7	2.47 ± 0.09	22.8 ± 1.1	trace
	over-ripe	4.3 ± 0.2	3.97 ± 0.11	10.6 ± 0.6	no activity
Chester Thornless	green	19.0 ± 1.9	2.00 ± 0.06	51.0 ± 2.4	16.0 ± 0.8
	pink	17.9 ± 1.5	1.94 ± 0.07	40.8 ± 1.3	2.1 ± 0.2
	ripe	15.5 ± 0.9	3.01 ± 0.10	33.7 ± 1.1	trace
	over-ripe	5.3 ± 0.1	5.78 ± 0.16	14.9 ± 0.8	no activity
Smoothstem	green	16.2 ± 1.4	2.10 ± 0.10	40.5 ± 2.1	14.0 ± 0.5
	pink	14.5 ± 1.1	1.95 ± 0.08	36.3 ± 1.9	1.6 ± 0.1
	ripe	12.9 ± 1.1	2.99 ± 0.25	29.9 ± 1.3	trace
	over-ripe	5.6 ± 0.5	4.89 ± 0.51	14.9 ± 0.5	no activity
Thornfree	green	16.0 ± 1.3	1.76 ± 0.09	39.1 ± 1.9	13.7 ± 0.4
	pink	14.0 ± 1.1	1.71 ± 0.05	35.1 ± 1.2	1.5 ± 0.1
	ripe	12.8 ± 0.6	2.84 ± 0.30	27.4 ± 1.4	trace
	over-ripe	5.9 ± 0.2	4.60 ± 0.39	12.9 ± 0.6	no activity
$LSD_{0.05}$		0.60	0.05	1.19	0.31
significance[b]					
cultivar [C]		**	**	**	**
maturity [M]		**	**	**	**
[C] × [M]		**	**	**	**

[a] Data expressed as mean ± SEM ($n = 3$). [b] **, significant at $p \leq 0.05$.

4 °C. The supernatants were used for GSH and GSSG determinations.

GSH and GSSG were assayed using the method described by Castillo and Greppin (*20*). Total glutathione equivalents were determined by reacting 0.1 mL of extract with 60 mM KH_2PO_4−2.5 mM EDTA buffer (pH 7.5), 0.6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 200 mM Tris-HCl (pH 8.0), 1 unit of GR (from spinach, EC 1.6.4.2), and 0.2 mM NADPH. The reaction was followed as the rate of change in absorbance at 412 nm, and the total glutathione content was calculated from a standard curve. GSSG was determined after removal of GSH from the sample extract. GSH was determined from the reaction mixture by mixing 0.1 mL of extract with 60 mM KH_2PO_4−2.5 mM EDTA buffer (pH 7.5) and 0.6 mM DTNB ′in 200 mM Tris-HCl (pH 8.0). The mixture was incubated at 30 °C for 10 min, and the reaction was followed as the rate of change in absorbance at 412 nm. GSSG was determined by subtraction of GSH from total glutathione.

Protein Determination. Protein was determined according to the method of Bradford (*21*), using bovine serum albumin (BSA) as a standard.

Extraction, Fractionation, and Analysis of Lipids. Triplicate fruit samples of 30 g of fresh weight were collected during each sampling time. Lipids were extracted, fractionated, and analyzed according to the procedures described by Wang and Faust (*22*). Purified lipids were separated into neutral and glyco- and phospholipid fractions by silicic acid column chromatography on 100−200-mesh Bio-Sil A (Bio-Rad Laboratories, Richmond, CA). Total fatty acids esterified to polar lipids were derivatized to fatty acids methyl esters (FAME) for flame ionization detection−gas chromatography (FID-GC) analysis. *n*-Heptadecanoic acid was included in all samples as an internal standard, and methyl heptadecanoate was used as an external standard. Individual FAMEs were identified by a comparison of peak areas with those of authentic standards (Supelco). This tentative identification of major polar lipid fatty acids was corroborated by further analysis of FAMEs by gas chromatography−mass spectrometry (GC-MS) (*22*). Total phospholipids were determined by the spectrophotometric assays of Ames (*23*).

The neutral lipid fraction from blackberry tissues was dried under a stream of N_2 and redissolved in 2 mL of hexane. Silicic acid column chromatography (100−200-mesh Bio-Sil A, Bio-Rad Laboratories) was used to separate the steryl ester and free sterols by elution with 10 hexane/1 ether (v/v; steryl ester) and 2 hexane/1 ether (v/v; free sterols). Steryl ester was cleaved to free sterol by saponification (1 M KOH in 80% ethanol for 1 h at 80 °C under N_2) and hexane extraction. Lathosterol (cholest-7-en-3β-ol) was added to each sample as an internal standard. Free sterols were precipitated with digitonin. The digitonides were collected on a glass fiber filter (Reeve Angel, Clifton, NJ) and washed with 1 acetone/2 diethyl ether (v/v). Free sterols were liberated from the digitonides by refluxing in 1 mL of pyridine for 30 min at 100 °C. After cooling, 1 mL of deionized, distilled water was added, and the free sterols were recovered by extraction with 2 mL of hexane. Quantification of sterol was determined by FID-GC.

Statistical Analysis. Data were subjected to analysis of variance, and means were compared by least significant difference (LSD). The effect of cultivar and maturity on oxygen-scavenging enzymes (SOD, G-POD, GSH-POD, and CAT), enzymes in the ascorbate−glutathione cycle (ascorbate-POD, MDAR, DHAR, and GR), and nonenzyme components in the ascorbate−glutathione cycle (AsA, DHAsA, GSH, and GSSG) were evaluated by Fisher's LSD multiple-comparison test used in NCSS (*24*). Differences at $p \leq 0.05$ were considered to be significant.

RESULTS

Activities of Oxygen-Scavenging Enzymes. Varying amounts of activity of oxygen-scavenging enzymes, SOD, G-POD, and GSH-POD, were detected in the fruit of different developmental stages of four thornless blackberry cultivars (Black Satin, Chester Thornless, Smoothstem, and Thornfree). CAT activity was detected in only the green and pink stages, and no activity was detected in the over-ripe stage of blackberries (Table 1). Cv. Chester Thornless consistently yielded the highest activity and cv. Black Satin contained the least activity among the different cultivars (Black Satin, Chester Thornless, Smoothstem, and Thornfree), for each of the enzymes tested. The activities of oxygen-scavenging enzymes, SOD, GSH-POD, and CAT, declined substantially as the fruits matured. The largest decrease in SOD activity occurred in Chester Thornless fruit, ranging from 19.0 units/mg of protein in the green stage to 5.3 units/mg of protein in over-ripe fruits. SOD activities in Smoothstem, Thornfree, and Black Satin were also reduced with fruit maturation from 16.2 to 5.6 units/

Table 2. Activities of AsA-POD, MDAR, DHAR, and GR in Thornless Blackberry Fruit at Different Stages of Maturity[a]

cultivar	maturity	AsA-POD (nmol/mg of protein·min)	MDAR (nmol/mg of protein·min)	DHAR (nmol/mg of protein·min)	GR (nmol/mg of protein·min)
Black Satin	green	159.7 ± 12	26.4 ± 1.8	11.5 ± 0.9	21.3 ± 1.7
	pink	137.1 ± 11	21.5 ± 1.5	9.9 ± 0.4	19.9 ± 1.4
	ripe	116.2 ± 9	18.0 ± 1.3	8.4 ± 0.4	15.6 ± 1.0
	over-ripe	35.9 ± 2	6.9 ± 1.0	3.1 ± 0.1	7.9 ± 0.4
Chester Thornless	green	222.1 ± 15	31.0 ± 2.1	14.6 ± 0.7	27.5 ± 1.9
	pink	187.2 ± 13	29.5 ± 2.2	12.9 ± 0.8	24.7 ± 1.7
	ripe	171.0 ± 13	28.9 ± 2.7	12.0 ± 0.7	22.8 ± 1.7
	over-ripe	59.8 ± 3	7.1 ± 0.4	5.8 ± 0.2	12.0 ± 1.0
Smoothstem	green	191.4 ± 15	30.0 ± 3.0	12.7 ± 0.8	24.9 ± 1.5
	pink	166.6 ± 11	28.2 ± 2.6	11.0 ± 0.8	21.6 ± 1.5
	ripe	159.2 ± 12	24.7 ± 2.2	10.9 ± 0.5	19.9 ± 1.3
	over-ripe	54.8 ± 5.6	7.0 ± 0.5	4.4 ± 0.2	11.0 ± 0.3
Thornfree	green	186.5 ± 19	29.1 ± 2.9	12.0 ± 0.5	23.6 ± 1.8
	pink	169.1 ± 16	27.8 ± 2.4	11.1 ± 0.4	20.2 ± 1.6
	ripe	151.9 ± 11	24.0 ± 2.1	10.6 ± 0.6	18.9 ± 1.5
	over-ripe	49.1 ± 2	9.8 ± 0.5	4.1 ± 0.2	9.5 ± 1.2
$LSD_{0.05}$		4.18	1.07	0.43	0.54
significance[b]					
cultivar [C]		**	**	**	**
maturity [M]		**	**	**	**
[C] × [M]		**	**	**	**

[a] Data expressed as mean ± SEM ($n = 3$). [b] **, significant at $p \leq 0.05$.

mg of protein, from 16.0 to 5.9 units/mg of protein, and from 13.8 to 4.3 units/mg of protein, respectively (Table 1). In all four thornless blackberry cultivars, the greatest drop in SOD activity occurred between the ripe and over-ripe stages (Table 1). GSH-POD activity also declined considerably as the fruits matured. GSH-POD activity in Chester Thornless fruit decreased from 51.0 in green fruits to 14.9 nmol/mg of protein·min in ripe fruits, whereas Black Satin, Smoothstem, and Thornfree cultivars contained significantly lower GSH-POD activity than Chester Thornless (Table 1). Chester Thornless blackberry contained the highest CAT activity at the green stage, with 16.0 units/mg of protein, whereas Black Satin fruits yielded the lowest, 12.5 units/mg of protein at the green stage. CAT activity diminished during ripening, and no enzyme activity could be detected in four thornless blackberry cultivars at the over-ripe stage (Table 1). SOD, GSH-POD, and CAT generally appeared to decrease as blackberry fruit matured, and guaiacol-POD activity increased as the fruit ripened. Guaiacol-POD activity increased from 2.00 ΔA/mg of protein·min in green fruits to 5.78 ΔA/mg of protein·min in over-ripe fruits of Chester Thornless. The most substantial increase in guaiacol-POD occurred between the ripe and over-ripe stages of all four thornless blackberry cultivars. Overall, Chester Thornless blackberry fruits contained the highest levels of oxygen-scavenging enzyme activities at all four maturity stages, followed by Smoothstem, Thornfree, and Black Satin (Table 1).

Activities of Enzymes in the Ascorbate—Glutathione Cycle. Activities of enzymes for scavenging hydrogen peroxide in the ascorbate−glutathione cycle also varied among different cultivars and different developmental stages (Table 2). Activities of these enzymes including AsA-POD, MDAR, DHAR, and GR declined as the fruits ripened (Table 2). For each of the enzymes, a steady decrease of activity from the green to the ripe stages and a large drop from the ripe to the over-ripe stage were detected. AsA-POD had the highest activity among the enzymes in the ascorbate−glutathione cycle. Chester Thornless fruit had the highest AsA-POD activity of the four thornless blackberry cultivars, with 222.1 nmol/mg of protein·min at the green stage declining to 59.8 nmol/mg of protein·min at the over-ripe stage. Black Satin had the lowest AsA-POD activity, lessened from 159.7 nmol/mg of protein·min in green fruit to the lowest activity in over-ripe fruit of 35.9 nmol/mg of protein·min. In Chester Thornless, MDAR activity in the fruit ranged from 31.0 nmol/mg of protein·min in green stage to 7.1 nmol/mg of protein·min in the over-ripe stage (Table 2). Chester Thornless fruit also contained higher DHAR and GR activities than Black Satin, Smoothstem, and Thornfree fruit. DHAR activity in Chester Thornless fruit ranged from 14.6 nmol/mg of protein·min in green fruits to 5.8 nmol/mg of protein·min in over-ripe fruits, whereas GR activity decreased from 27.5 nmol/mg of protein·min in green fruits to 12.0 nmol/mg of protein·min in over-ripe fruits. Chester Thornless consistently showed the highest activity for the enzymes in the ascorbate−glutathione cycle, whereas Black Satin had the lowest activity compared to the other cultivars.

Nonenzyme Components in the Ascorbate—Glutathione Cycle. *AsA and DHAsA.* Different blackberry cultivars also contained different amounts of the nonenzyme components in the ascorbate−glutathione cycle during different developmental stages (Table 3). In green fruits, AsA contents ranged from 2.12 μmol/g of fwt in Black Satin to 3.00 μmol/g of fwt in Chester Thornless. These nonenzyme components also decreased as the fruits matured. AsA levels decreased greatly between green and pink stages and continued to steadily decline from the pink to over-ripe stages. In Chester Thornless blackberries, AsA content dropped from 3.00 μmol/g of fwt in the green fruit to 1.55 μmol/g of fwt in the pink fruit and was 0.94 μmol/g of fwt at the over-ripe stage (Table 3). The highest DHAsA level was at 0.20 μmol/g of fwt for Chester Thornless fruit, whereas the lowest DHAsA content occurred in Black Satin, with 0.10 μmol/g of fwt. The DHAsA levels decreased slowly from the green to the over-ripe stages. At the green stage Chester Thornless fruit contained 0.20 μmol/g of fwt, whereas at the over-ripe stage the DHAsA content

Table 3. Activities of AsA, DHAsA, GSH, and GSSG in Thornless Blackberry Fruit at Different Stages of Maturity[a]

cultivar	maturity	AsA (μmol/g of fwt)	DHAsA (μmol/g of fwt)	GSH (nmol/g of fwt)	GSSG (nmol/g of fwt)
Black Satin	green	2.12 ± 0.21	0.17 ± 0.01	70.8 ± 2.1	14.7 ± 1.4
	pink	0.99 ± 0.07	0.14 ± 0.01	66.6 ± 4.8	14.4 ± 1.2
	ripe	0.70 ± 0.05	0.12 ± 0.01	60.4 ± 4.4	13.3 ± 0.9
	over-ripe	0.42 ± 0.01	0.10 ± 0.01	19.0 ± 1.5	8.5 ± 0.8
Chester Thornless	green	3.00 ± 0.51	0.20 ± 0.02	99.3 ± 7.0	19.6 ± 2.1
	pink	1.55 ± 0.20	0.18 ± 0.02	85.9 ± 7.7	18.0 ± 1.7
	ripe	1.30 ± 0.22	0.17 ± 0.01	79.0 ± 5.9	17.0 ± 1.6
	over-ripe	0.94 ± 0.06	0.16 ± 0.01	23.4 ± 1.1	10.1 ± 0.9
Smoothstem	green	2.25 ± 0.55	0.16 ± 0.01	86.3 ± 7.7	17.4 ± 1.1
	pink	1.00 ± 0.04	0.14 ± 0.02	80.9 ± 7.1	16.9 ± 1.3
	ripe	0.85 ± 0.07	0.13 ± 0.01	78.4 ± 6.6	16.9 ± 1.5
	over-ripe	0.61 ± 0.04	0.12 ± 0.01	25.1 ± 2.2	10.8 ± 0.9
Thornfree	green	2.15 ± 0.42	0.16 ± 0.01	75.5 ± 6.7	16.0 ± 1.1
	pink	1.03 ± 0.24	0.14 ± 0.01	70.9 ± 6.1	15.2 ± 1.2
	ripe	0.81 ± 0.09	0.12 ± 0.01	64.1 ± 5.8	14.1 ± 1.2
	over-ripe	0.51 ± 0.03	0.12 ± 0.01	22.2 ± 1.9	10.0 ± 0.8
$LSD_{0.05}$		0.034	0.012	3.26	0.81
significance[b]					
cultivar [C]		**	**	**	**
maturity [M]		**	**	**	**
[C] × [M]		**	**	**	**

[a] Data expressed as mean ± SEM ($n = 3$). [b] **, significant at $p \leq 0.05$.

Table 4. Ratios of AsA/DHAsA and GSH/GSSG in Thornless Blackberry Fruit at Different Stages of Maturity[a]

cultivar	maturity	AsA/DHAsA	GSH/GSSG
Black Satin	green	12.47	4.83
	pink	7.92	4.62
	ripe	5.87	4.53
	over-ripe	3.96	2.23
Chester Thornless	green	15.00	5.07
	pink	8.61	4.77
	ripe	7.48	4.65
	over-ripe	5.89	2.33
Smoothstem	green	14.06	4.94
	pink	6.99	4.76
	ripe	6.31	4.63
	over-ripe	5.08	2.31
Thornfree	green	13.17	4.71
	pink	7.49	4.66
	ripe	6.76	4.56
	over-ripe	4.29	2.22
$LSD_{0.05}$		0.670	0.171
significance[b]			
cultivar [C]		**	**
maturity [M]		**	**
[C] × [M]		**	**

[a] Data expressed as mean ± SEM ($n = 3$). [b] **, significant at $p \leq 0.05$.

was 0.16 μmol/g of fwt. Black Satin, Smoothstem, and Thornfree cultivars followed a similar pattern. The highest AsA/DHAsA ratio occurred at the green stage in Chester Thornless fruit. This ratio showed the greatest decrease from the green to pink stages in all four cultivars and further declined as the fruit matured to the over-ripe stage (Table 4).

GSH and GSSG. Blackberries have lower contents of GSH and GSSG than of AsA and DHAsA among the cultivars (Table 3). GSH contents ranged from 99.3 nmol/g of fwt in the green fruit of Chester Thornless to 19.0 nmol/g of fwt in the over-ripe fruit of Black Satin. Similarly, the lowest GSSG content was found in the over-ripe fruit of Black Satin, which had ~8.50 nmol/g of wt, whereas the highest GSSG content was found in green fruit of Chester Thornless berries, which had 19.6 nmol/g of fwt. GSH and GSSG contents both lessened from the green to ripe stages. In Chester Thornless, GSH levels dropped from 99.3 nmol/g of fwt in green fruits to 79.0 nmol/g of fwt in ripe fruits, whereas GSSG contents dropped from 19.6 to 17.0 nmol/g of fwt. The greatest decrease in GSH and GSSG contents occurred between the ripe and over-ripe stages. Over-ripe fruits in the Chester Thornless berries yielded a GSH content of only 23.4 nmol/g of fwt and a GSSG content of 10.1 nmol/g of fwt. Chester Thornless yielded the highest ratios of GSH/GSSG compared to the four blackberry cultivars. The GSH to GSSG ratio for green Chester Thornless fruits was 5.07 and decreased as the fruits matured. Among the cultivars tested, Chester Thornless also yielded the highest amount for each nonenzyme component in the ascorbate−glutathione cycle, followed by Smoothstem, Thornfree, and Black Satin.

Membrane Lipids and Sterols. Fatty acids of blackberry fruits in phospholipids included mainly palmitate (C16:0), stearate (C18:0), oleate (C18:1), linoleate (C18:2), and linolenate (C18:3). Blackberry fruits had higher ratios of unsaturated fatty acid (18:1 + 18:2 + 18:3) to saturated fatty acid (16:0 + 18:0) of phospholipids in the green stage and decreased as the fruit ripened (Table 5).

The free sterols in blackberry fruit are β-sitosterol, cholesterol, and campesterol. β-Sitosterol is the major desmethylsterol in these four thornless cultivars of blackberry fruit, comprising >90% of the sterol present. Cholesterol and campesterol are present in ca. 2 and 4%, respectively. Stigmasterol was not detected. The ratio of free sterols to phospholipids was higher in more ripened fruit tissue (Table 5). The results of the lipid studies indicate a decrease in the fluidity of membranes in ripening or senescence fruit tissue. Chester Thornless blackberry fruit contained the highest ratios of unsaturated (18:1 + 18:2 + 18:3) to saturated (16:0 + 18:0) fatty acids of phospholipids and the lowest ratios of sterols to phospholipids, followed by Smoothstem, Thornfree, and Black Satin at all four maturity stages (Table 5).

Table 5. Ratios of Unsaturated Fatty Acid (18:1 + 18:2 + 18:3) to Saturated Fatty Acid (16:0 + 18:0) and Free Sterols to Phospholipids in Thornless Blackberry Fruit at Different Stages of Maturity[a].

cultivar	maturity	unsaturated fatty acid/ saturated fatty acid	free sterols/ phospholipids
Black Satin	green	3.40	0.046
	pink	3.14	0.055
	ripe	2.13	0.070
	over-ripe	1.02	0.097
Chester Thornless	green	3.76	0.038
	pink	3.50	0.045
	ripe	2.76	0.061
	over-ripe	1.25	0.086
Smoothstem	green	3.58	0.039
	pink	3.37	0.048
	ripe	2.59	0.064
	over-ripe	1.20	0.088
Thornfree	green	3.47	0.044
	pink	3.16	0.058
	ripe	2.26	0.071
	over-ripe	1.15	0.094
$LSD_{0.05}$		0.20	0.005
significance[b]			
cultivar [C]		**	**
maturity [M]		**	**
[C] × [M]		**	**

[a] Data expressed as mean ± SEM ($n = 3$). [b] **, significant at $p \leq 0.05$.

DISCUSSION

Free radicals are formed during normal cell metabolism. They can be generated enzymatically through mitochondrial respiration or the cytochrome P450 system, by various soluble enzymes, and also from nonenzymatic reactions of oxygen with organic compounds (*25*). Increased levels of free radicals or active oxygen species (i.e., $O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$, and $^1O_2$) create oxidative stress, which leads to a variety of biochemical and physiological lesions often resulting in metabolic impairment. In normal circumstances, concentrations of these oxygen radicals are likely to remain low because plants have an efficient enzymatic system for active oxygen detoxification that includes SOD, G-POD, GSH-POD, CAT, enzymes of the ascorbate−glutathione cycle (AsA-POD, MDAR, DHAR, and GR), and nonenzyme antioxidants (AsA, DHAsA, GSH, GSSG, α-tocopherol, β-carotene, and phenolics) (*26*−*28*).

Various activities of the oxygen-scavenging enzymes, SOD, G-POD, GSH-POD, and CAT, were detected in blackberry fruit during different developmental stages. Chester Thornless consistently yielded the greatest and Black Satin contained the least activity among the four different cultivars for each of the enzymes tested. The highest SOD, GSH-POD, and CAT activities were seen at the green stage and generally appeared to decrease as blackberry fruit matured. However, G-POD activity increased as fruit ripened. The exact cause of this increase in G-POD activity is not known. It could be related to the increase in oxidative stress during maturation and ripening. G-POD is involved in a large number of biochemical and physiological processes and may change quantitatively and qualitatively during growth and development (*29*). High G-POD activity in over-ripe fruits may have resulted from the inhibition of the catalase. Increased G-POD activity toward the end of ripening of saskatoon (*Amelanchier alnifolia* Nutt.) has also been reported (*6*). Low levels of SOD, GSH-POD, and CAT activities may lead to a high level of H_2O_2 in fruit, resulting in a loss of membrane integrity.

SOD has been detected in a wide range of living organisms (*30*). SOD catalyzes the breakdown of $O_2^{\bullet -}$ to O_2 and H_2O_2, removes singlet oxygen as well as $O_2^{\bullet -}$, prevents formation of OH^- (*31*), and has been implicated as an essential defense against the potential toxicity of oxygen (*32*). However, the defensive action of SODs against $O_2^{\bullet -}$ shows age-related changes (*33*). Our data support other reports that SOD activity decreases with senescence of *Nicotiana tabacum* and *Dianthus caryophyllus* (*33*, *34*) and ripening of tomato fruit (*9*). CAT is an antioxidant enzyme. It has been reported that catalase functions in ensuring the removal of H_2O_2, supplying free O_2, and detoxifying harmful metabolic products. The H_2O_2 formed by G-POD may be scavenged by CAT. GSH-POD also scavenges H_2O_2, catalyzes the peroxidation of GSH, and forms the oxidized disulfide form of glutathione (GSSG) as a product (*35*). Decreased GSH-POD and CAT activities as fruit ripens result in decreased H_2O_2-scavenging capacity.

Ascorbic acid is an essential compound in plant tissues and has been the focus of numerous studies in relation to the enzymatic or nonenzymatic oxidation reactions in the biological system. Ascorbic acid has been reported to act as an antioxidant and functions as a cosubstrate of plant peroxidases, such as the AsA-POD system that produces DHAsA (*36*). DHAsA is reduced to ascorbic acid by MDAR or DHAR at the expense of NADH and GSH. AsA-POD uses ascorbate as an electron donor, but the basic properties of AsA-POD are very different from those of G-POD with regard to their amino sequences and other molecular properties (*37*).

Glutathione is generally considered to be ubiquitous in living cells. Glutathione often exists in both a reduced form (GSH) and an oxidized form, GSSG. GSH plays an important role in the stabilization of many enzymes. It also has a more general role as an oxidant scavenger by serving as a substrate for DHAR and reacting directly with free radicals including the hydroxyl radical to prevent the inactivation of enzymes by oxidation of the essential thiol group (*38*). The majority of glutathione in the cell is maintained in the reduced state. GSH represented the largest fraction of non-protein thiol. Depending on the stage of fruit development, its concentration may range from 99.3 to 19.0 nmol/g of fwt for green and over-ripe fruit of blackberry, respectively. GSSG was found to be present only in small quantities. A high GSH/GSSG ratio is necessary for several physiological functions. This includes activation and inactivation of redox-dependent enzyme systems (*39*) and regeneration of the cellular antioxidant ascorbic acid under oxidative conditions (*40*).

GR is a ubiquitous NADPH-dependent enzyme and present in cells of both plants and animals (*41*). It has been suggested that in higher plants GR may be a rate-limiting enzyme for defense against active O_2 toxicity (*42*, *43*). In blackberry fruit, we found that decreased GR activity was associated with maturation and ripening. GR seems to be mainly located in the cytosol and catalyzes the NADPH-dependent reduction of GSSG to the reduced form (GSH) (*41*) and may play an important

role in regulating fruit ripening. High activity of GR and high levels of GSH were found when fruit were in the green stage.

There was a corresponding increase in the percentage of saturated lipids in ripening fruit tissue. The ratios of unsaturated (18:1 + 18:2 + 18:3) to saturated (16:0 + 18:0) fatty acids of phospholipids were lower in ripe and over-ripe fruit tissue than in green fruit tissue. This result perhaps reflects the depletion of unsaturated fatty acids by lipid peroxidation. Fatty acid unsaturation is one of the key factors regulating membrane function (*44*). The decrease of this ratio may contribute to the decrease in membrane fluidity in advanced ripening fruit.

A high content of sterols and sterol esters is characteristic for the plasma membrane of plant cells. These steryl lipids serve to stabilize the lipid bilayer. Changes in sterol level and composition have been shown to affect membrane permeability and development in higher plants (*45*). It was found that a large increase in the content of steryl lipids, as well as a dramatic change in their sterol composition, occurs with ripening in apple (*46*) and tomato (*47*). The lipid composition of various membranes in plant cells greatly affects the fluidity of their lipid matrix (*48*). The interaction of free sterols with phospholipids could affect membrane function and permeability (*49*). The ratio of free sterols to phospholipids was higher, whereas the ratios of unsaturated to saturated fatty acids was lower, in over-ripe blackberry fruit tissue. These results indicate a decrease in the fluidity of membranes in over-ripe fruit.

Reduced membrane damage has been linked to increased enzymatic defenses against oxygen radicals, together with synthesis of free radical scavengers (*27*). High levels of antioxidant enzymes have been correlated with delayed senescence in fruits (*8*). Our data showed that the maturation and ripening of the fruit from these four blackberry cultivars (Chester Thornless, Smoothstem, Thornfree, and Black Satin) were accompanied by a decrease in the activities of oxygen-scavenging enzymes (SOD, GSH-POD, and CAT), enzymes in the ascorbate−glutathione cycle (AsA-POD, MDAR, DHAR, and GR), and nonenzyme components in the ascorbate−glutathione cycle such as AsA, DHAsA, GSH, and GSSG and the ratios of AsA/DHAsA and GSH/GSSG. The low enzyme activities and low antioxidant content occur concomitantly with increases in the saturation of fatty acids in polar lipids and higher ratios of sterols to phospholipids. These events may result in increased oxidative stress and lipid peroxidation and hasten many metabolic changes associated with maturation and ripening in blackberry fruit.

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