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Measuring Flavonoid Enzyme Activities in Tissues of Fruit Species

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Flavonoids are important secondary metabolites, which are ubiquitously present in plant-derived food. Since flavonoids may show beneficial effects on human health, there is increasing interest in the availability of plants with a tailor-made flavonoid spectrum. Determination of flavonoid enzyme activities and investigations into their substrate specificity are an important precondition for both classical and molecular approaches. We tested two different protocols for enzyme preparation from eight fruit species. In many cases, a protocol adapted for polyphenol-rich tissues was superior. Using a suitable protocol for investigations of kiwi fruits, we show that flavanone 3-hydroxylase is absent in the green-fleshed cultivar Hayward. As flavonoid enzyme activities could be detected in harvested kiwi fruits over a storage period of five months, postharvest modification of the flavonoid spectrum has to be expected.

KEYWORDS: Blackberry (*Rubus fruticosa*); cherry (*Prunus avium*); sour cherry (*Prunus cerasus*); raspberry (*Rubus* x *ideaus*); gooseberry (*Ribes uva-crispa*); elder (*Sambucus* x *nigra*); kiwi fruit (*Actinidia deliciosa*); plum (*Prunus domestica*); flavonoid biosynthesis; enzyme preparation; polyphenol (tannin) rich tissue

INTRODUCTION

Flavonoids are important bioactive compounds, which fulfill a broad range of physiological functions *in planta* such as pollinator and bird attraction, UV and light protection, metal chelation, herbivore deterrence, and pathogen defense (*1*, *2*). They are ubiquitously present in plants and therefore also in human food. The nutritional relevance of flavonoids is demonstrated by an impressive spectrum of health-related effects such as reducing the incidence of cardiovascular diseases, cancer, hyperlipidemias, and other chronic diseases (*3*−*5*). Therefore, there is a growing interest in the availability of fruit crops showing an optimal flavonoid concentration and composition for specific purposes. This may be achieved either by exploring the genetic diversity of existing varieties or by pathway engineering for a tailor-made flavonoid spectrum. In both cases, the availability of suitable protocols for the determination of flavonoid enzyme activities is a prerequisite. The knowledge of the presence or absence of selected flavonoid enzymes and of their substrate specificities allows us to predict resulting phenotypes (*6*) and to define the necessary steps for a pathway engineering approach.

Large parts of the flavonoid pathway are well-established at the level of enzymes and genes (**Figure 1**). The earliest experiments were performed with a parsley cell culture in which flavonoid biosynthesis could be induced by UV-light (*7*). Later, flower buds were revealed to be optimal sources for enzymatic studies of the flavonoid pathway. The high enzyme activities of buds are probably based on the rapid flower development process and the large amounts of anthocyanins and other flavonoid classes formed in petal tissues. In addition, the availability of genetically defined plant material rapidly increased the level of knowledge of the flavonoid metabolism and the involved enzymes and genes. The flavonoid pathway was studied in a broad range of economically important ornamental plants (*7*), and the creation of new varieties with unusual flower color is an attractive biotechnological strategy (*6*). A prominent example of successful flavonoid pathway engineering is the establishment of blue carnations, which was primarily based on the knowledge of flavonoid enzymes and their substrate specificities in this flower species (*8*, *9*).

Suitable enzyme preparations can be obtained from many plant tissues with a relatively simple protocol (*10*−*16*), which is based on the maceration of the cell tissue in the presence of cell wall-disrupting quartz sand, polyphenol-binding polyclar AT (insoluble polyvinyl pyrrolidone), and sodium ascorbate as antioxidative agent in the extraction buffer. In addition, low-molecular compounds such as polyphenols, metal ions, and interfering cofactors are removed by a gel chromatography step. More recalcitrant plant materials, which may contain high amounts of polyphenols, tannins, and glucanes, require quite sophisticated protocols to overcome the presence of disturbing plant ingredients (*17*, *18*). In 1992, Claudot and Drouet published a protocol that was optimized for the measurement of chalcone synthase (CHS) in polyphenol-rich walnut tissues (*19*). Furthermore, an

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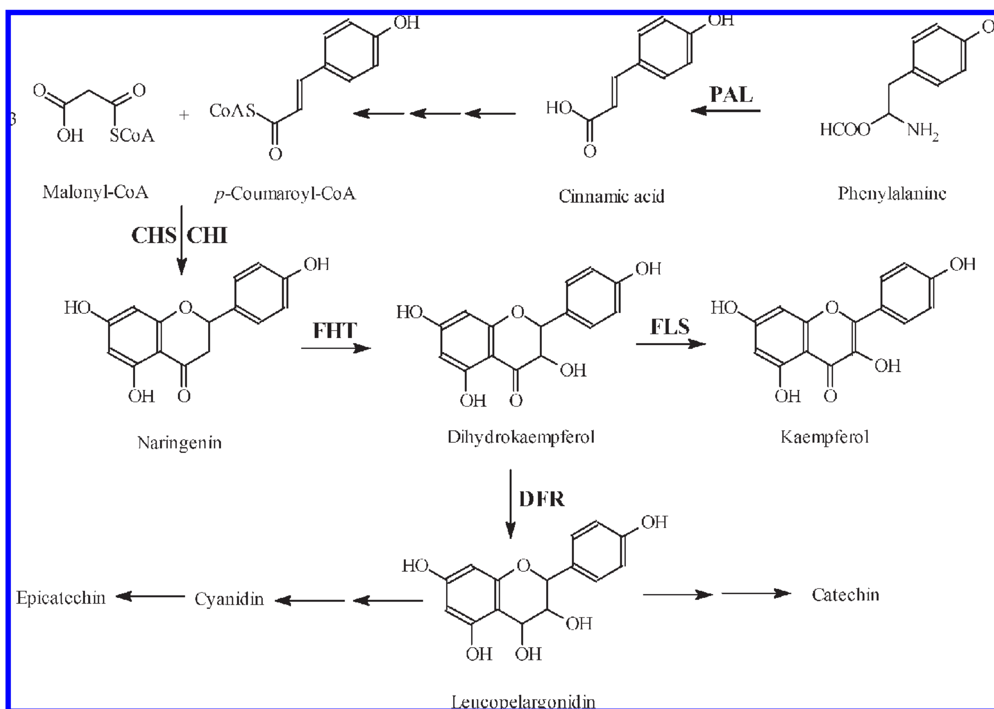

Figure 1. Selected part of the flavonoid pathway showing the investigated enzyme activities.

adapted protocol for the dihydroflavonol 4-reductase (DFR) assays in lignocellulosic tissues is available (*20*). These protocols basically rely on oxygen removal from the extraction buffer, removal of polyphenols with polyphenol binding agents, and on the presence of relatively high amounts of antioxidative protectants in the extraction buffer.

We investigated two different protocols (*12*, *19*) for their suitability to provide enzyme preparations of sufficient quality for enzymatic studies with phenylalanine ammonia lyase (PAL), chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), DFR, and flavonol synthase (FLS). Up to four different plant tissues (leaves, unripe and ripe fruits, and flowers) from eight fruit crops were tested. It was shown that a protocol adapted from Claudot and Drouet (*19*) is superior in many cases. From these results, we were able to demonstrate the absence of FHT in kiwi fruits of the commercially important cultivar Hayward. In addition, we demonstrated that the flavonoid metabolism is active in kiwi fruits after harvest and even after several months of storage.

MATERIALS AND METHODS

General. TLC was performed on Merck precoated cellulose plates (without fluorescence indicator, 1.0571.001, Darmstadt, Germany); radiolabeled substances were detected with a Berthold LB 2842 TLC Linear Analyzer (Wildbad, Germany) and a Winspectral 1414 scintillation counter (Perkin-Elmer, Vienna, Austria).

Plant Material. Fruits of *Prunus avium* cv. Augustkirsche, *Prunus cerasus* cv. Pandy 114, and *Prunus domestica* were obtained from the experimental orchard of the Institute of Horticulture and Viticulture (University of Natural Resources and Applied Life Sciences, Vienna, Austria). Fruits and leaves of *Rubus idaeus*, *Ribes uva-crispa*, and *Sambucus nigra* were collected in a private garden in 8564 Krottendorf-Gaisfeld (Styria, Austria), the samples of *Rubus fruticosa* were collected in the forests of the same area. Ripe fruits were harvested at technical ripeness, and unripe fruits were still uncolored and showed approximately 50% of the size of ripe fruits; leaf samples were collected from young enrolling leaves. The plant material was harvested in 2002, shock-frozen in liquid nitrogen, and stored at −80 °C. Kiwi fruits (*Actinidia deliciosa*, cv. Hayward) were harvested in 2006 from a commercial orchard (Faenza, Italy), kept for 72 h at 15 °C, and stored at −0.8 °C in controlled atmosphere (RH 92−95%, ethylene below 0.02 ppm, O_2 1.8−2%, CO_2 3−4.5%). Shock-frozen fruits were ground in a mill; leaves were ground in liquid nitrogen in a mortar.

Chemicals. [2-^{14}C]-Malonyl-coenzyme A (2 GBq/mmol) and L-[U-^{14}C]phenylalanine (17 GBq/mmol) were obtained from Amersham International (Freiburg, Germany). [^{14}C]-Naringenin (0.8 GBq/mmol), [^{14}C]-eriodictyol, [^{14}C]-dihydrokaempferol (0.8 GBq/mmol), and [^{14}C]-dihydroquercetin (0.8 GBq/mmol) were prepared as recently described (*13*). Sephadex G25 (medium) was purchased from GE Healthcare (Munich, Germany). Dowex 1 × 2, NADPH, EDTA, BSA, and DIECA were obtained from Sigma-Aldrich (Vienna, Austria) and L-cysteine and PEG 20000 from VWR International (Vienna, Austria).

Buffers Used. The following buffers were used for enzyme preparation. Extraction buffer 1: 0.1 M Tris/HCl at pH 7.5 containing 0.4% sodium ascorbate. Extraction buffer 2: 0.7 M KH_2PO_4/K_2HPO_4 at pH 8.0 containing 0.4 M sucrose, 0.4 M sodium ascorbate 1 mM $CaCl_2$, 30 mM EDTA, 50 mM cysteine, 50 mM DIECA, 1.5% PEG 20000, and 0.1% BSA. To remove dissolved oxygen, extraction buffer 2 was kept at 100 °C for 10 min and cooled under N_2 atmosphere before the addition of cysteine, DIECA, PEG 20000, and BSA. The following buffers were used for enzyme preparation: buffer 1 (PAL), 0.1 M H_3BO_3 at pH 8.5 containing 0.4% sodium ascorbate; buffer 2 (CHS), 0.1 M Tris/HCl at pH 8.25 containing 0.4% sodium ascorbate; buffer 3 (FHT, FLS), 0.1 M Tris/HCl at pH 7.5 containing 0.4% sodium ascorbate; buffer 4 (DFR), 0.1 M KH_2PO_4/K_2HPO_4 at pH 6.8 containing 0.4% sodium ascorbate.

Enzyme Preparations. Protocol 1 (*12*): 0.5 g of plant material, 0.25 g of quartz sand, 0.25 g of Polyclar AT, and 3 mL of extraction buffer 1 were homogenized in a precooled mortar. The homogenate was centrifuged for 10 min at 4 °C and 10000*g*.

Protocol 2 (*19*): 0.5 g of plant material and 0.5 g of Polyclar AT were homogenized in a precooled mortar, transferred to a falcon tube containing 0.5 g of Dowex (1 × 2) in 5 mL extraction buffer 2, and kept under nitrogen atmosphere for 15 min with weak stirring. The homogenate was filtered through glass wool and centrifuged for 20 min at 4 °C and 38000*g*.

To remove low molecular compounds, 400 μL of the supernatants obtained with both protocols were passed through a Sephadex G25 gel chromatography column. Protein content was determined by a modified Lowry procedure (*21*) using crystalline BSA as a standard. All data represent an average of at least three independent experiments.

Enzyme Assays. In a final volume of 100 μL, the PAL assay contained 40 μL of enzyme preparation (0.8−318 μg total protein), 5 μL of [^{14}C]-phenylalanine (1 nmol, 870 Bq), and 55 μL of buffer 1; the CHS/CHI assay

contained 40 μL of enzyme preparation (0.8−318 μg total protein), 5 μL of [^{14}C]-malonyl-CoA (1.5 nmol, 1300 Bq), 5 μL of *p*-coumaroyl-CoA (1.0 nmol), and 50 μL of buffer 2; the FHT assay contained 0.046 nmol of [^{14}C]-naringenin (108 Bq), 30 μL of enzyme preparation (0.6−239 μg total protein), 5 μL of 3.48 mM 2-oxoglutarate, 5 μL of 5 mM $FeSO_4 \cdot 7 H_2O$, and 80 μL of buffer 3; and the FLS assay contained 0.046 nmol of [^{14}C]-dihydrokaempferol (108 Bq), 40 μL of enzyme preparation (0.8−318 μg total protein), 5 μL of 3.48 mM 2-oxoglutarate, 5 μL of 2 mM $FeSO_4 \cdot 7 H_2O$, and 50 μL of buffer 3. In a final volume of 50 μL, the DFR assay contained 0.046 nmol of [^{14}C]-dihydroquercetin (108 Bq), 15 μL of enzyme preparation (1−3.6 μg total protein), 5 μL of 5.21 mM NADPH, and 35 μL of buffer 5. The assays were incubated for 30 min at 30 °C. PAL and CHS/CHI assays were stopped with 200 μL of ethyl acetate and 10 μL of acetic acid, and the amounts of product formed were determined on a scintillation counter. FHT, DFR, and FLS assays were terminated by the addition of 70 μL of ethyl acetate and 10 μL of acetic acid. To FLS assays, 10 μL of 0.1 mM EDTA was also added before the extraction. The organic phases were transferred to a precoated cellulose plate (Merck, Germany). After development of the TLC plates in chloroform/acetic acid/H_2O (10:9:1, v/v/v), conversion rates were determined with a TLC linear analyzer. All products were identified as described with authentic substances (*22*).

RESULTS/DISCUSSION

Determination of Enzyme Activities in Different Fruit Tissues. The activities of five key enzymes from the flavonoid pathway were tested in tissues from selected fruits by using enzyme preparations obtained with two different protocols. One was a relatively simple protocol, which is frequently used for flowers (*12*); the second was based on a protocol optimized for polyphenol-rich tissues (*19*). The investigations included PAL, CHS/CHI, FHT, DFR, and FLS (**Figure 1**). PAL, catalyzing the deamination of the amino acid phenylalanine, is an important enzyme that represents the interface between primary and secondary metabolism. The resulting cinnamic acid is the precursor for the biosynthesis of flavonoids, lignin, stilbenes, and coumarines. CHS is located at the entry to the flavonoid pathway providing 6′-hydroxychalcones as the first C15-structure, which are the intermediates for all flavonoid classes (**Figure 1**). FHT and FLS are dioxygenases that require 2-oxoglutarate, Fe^{2+}, and ascorbate as cofactors and catalyze the formation of dihydroflavonols and flavonols, respectively. DFR is an NADPH-dependent oxidoreductase, which reduces the oxo-group in position 4 to provide flavan 3,4-diols as the precursors for the formation of catechins, anthocyanidins, and epicatechins (**Figure 1**).

The aim was to identify a protocol that is suitable for the simultaneous determination of the five flavonoid enzymes. The procedure of Claudot and Drouet (*19*), however, showed very good results only with CHS/CHI and DFR but did not allow us to measure any FHT or FLS activity. This was presumably a result of the high EDTA concentration in the original preparation buffer, which masked Fe^{2+} and thereby decreased the availability of this cofactor for the dioxygenases. Therefore, the Fe^{2+} concentration in the FHT and FLS assays was increased to 5 mM to compensate for residual complexing agents present in the enzyme preparation after the gel chromatography step. In addition, the preparation buffer was modified by decreasing the EDTA concentration from 50 mM to 30 mM. This enabled the measurement of the dioxygenases but did not affect the other target enzymes. With a few exceptions, the enzyme preparations obtained with protocol 2 had a 2−10 times higher total protein content (**Table 1**) measured with the modified Lowry procedure (*21*), although the ratio of plant material/extraction buffer was 60% lower compared to that of protocol 1. The higher content of antioxidative compounds and other protective agents present in extraction buffer 2 presumably results in lower protein degradation during enzyme preparation. Specific activities for the flavonoid enzymes obtained with the two protocols are shown in **Table 1**. However, specific activities may provide a distorted picture particularly when the enzyme preparations contain low protein concentrations. This may result in high specific activities even if the amounts of product formed are low or close to the detectability limit. In these cases, conversion rates or product formation are more conclusive (**Table 2**). For FHT, DFR, and FLS, the

Table 1. Total Protein Contents of the Enzyme Preparations Obtained with Two Different Protocols and Resulting Specific Activities [nkat/kg Total Protein] of the Flavonoid Enzymes in the Investigated Fruit Tissues

		protein content [μg/μL]		PAL activity [nkat/kg]		CHS activity [nkat/kg]		FHT activity [nkat/kg]		DFR activity [nkat/kg]		FLS activity [nkat/kg]	
plant	tissue	protocol 1	protocol 2	protocol 1	protocol 2	protocol 1	protocol 2	protocol 1	protocol 2	protocol 1	protocol 2	protocol 1	protocol 2
blackberry	leaves	0.14	1.21	0	2	0	507	198	198	0	1630	259	259
	unripe fruits	0.09	0.78	0	3	0	916	0	440	0	3846	934	879
	ripe fruits	0.12	0.72	0	3	0	11956	388	443	332	5651	748	208
cherry	leaves	0.04	0.18	0	0	178	345	758	606	3409	3636	1136	1591
	ripe fruits	0.43	0.83	0	8	306	99	932	357	1491	1724	210	280
gooseberry	leaves	0.10	0.30	0	325	0	12095	0	1933	0	4000	300	200
	unripe fruits	0.34	1.10	0	77	17	5116	196	1039	0	2618	118	250
	ripe fruits	0.20	0.70	0	6	0	0	0	131	0	2741	0	465
elder	leaves	0.84	1.64	107	35	78	0	690	0	452	0	226	0
	unripe fruits	0.77	1.30	3	0	24	54	242	43	91	285	45	117
	ripe fruits	0.79	1.20	101	6	685	535	688	484	76	866	25	108
	flowers	8.09	7.95	25	19	61	9	64	46	19	16	12	10
kiwi fruit	leaves	0.61	0.56	198	0	62	0	0	0	649	0	82	33
	ripe fruits	2.88	1.08	280	47	269	8	0	0	104	31	14	9
plum	leaves	1.14	4.98	3	1	0	0	316	0	140	79	22	22
	unripe fruits	0.08	0.75	0	0	0	1167	0	833	0	4375	375	1000
	ripe fruits	0.04	0.52	0	14	0	798	0	0	1367	911	683	797
raspberry	leaves	0.13	0.85	0	66	0	3980	0	1105	0	1579	0	316
	unripe fruits	0.21	1.20	0	0	0	118	0	0	0	238	0	238
	ripe fruits	0.15	1.00	50	18	0	626	0	1537	0	2576	0	407
sour cherry	leaves	0.20	1.101	0	3	0	71	0	0	0	0	25	100
	unripe fruits	0.05	1.70	0	4	0	597	0	4400	1200	6600	400	1000
	ripe fruits	1.02	1.50	0	19	0	8870	15072	15652	7826	35217	4348	3261

Table 2. Activities of Selected Flavonoid Enzymes in Fruit Tissues by Use of Enzyme Preparations Obtained with Two Different Protocols[a]

		PAL		CHS/CHI		FHT		DFR		FLS	
plant	tissue	protocol 1	protocol 2	protocol 1	protocol 2	protocol 1	protocol 2	protocol 1	protocol 2	protocol 1	protocol 2
blackberry	leaves	−	+	−	++	+	+	−	++	+	+
	unripe fruits	−	+	−	++	−	+	−	++	++	++
	ripe fruits	−	+	−	+++	+	+	+	+++	++	+
cherry	leaves	−		−	+	+	+	++	++	+	+
	ripe fruits	−	++	++	++	+++	++	+++	+++	++	++
gooseberry	leaves	−	+++	−	+++		++	−	++	+	+
	unripe fruits	−	++	−	+++	+	+++	−	+++	+	++
	ripe fruits	−	+	−	−	−	+	−	+++	−	++
elder	leaves	+++	++	++	−	+++		++	−	++	−
	unripe fruits	+	−	+	++	++	+	+	++	+	++
	ripe fruits	++	++	++	++	+++	+++	++	+++	+	++
	flowers	+++	+++	++	++	+++	+++	++	++	++	++
kiwi fruit	leaves	++	++	++	−	−	−	++	+	+	+
	ripe fruits	+++	++	++	+	−	−	++	+	+	+
plum	leaves	++	+	−	−	+++	−	++	+	+	+
	unripe fruits	−	−	−	++	−	+	−	++	+	++
	ripe fruits	−	++	−	++	−	−	+	+	+	+
raspberry	leaves	−	++	−	++	−	++	−	++	−	+
	unripe fruits	−	−	−	+	−	−	−	+	−	+
	ripe fruits	++	++	−	++	−	++	−	++	−	++
sour cherry	leaves	−	+	−	+	−	−	−	−	−	+
	unripe fruits	−	++	−	+	−	++	+	++	+	++
	ripe fruits	−	++	−	++	+++	+++	++	+++	++	++

[a] For FHT, DFR, and FLS, the categories are based on observed conversion rates: 0−3%, −; 4−15%, +; 16−50%, ++;, 51−100, +++. For PAL and CHS/CHI, the categories are based on the amount of product formed: below the threshold of 1.3 Bq, −; 1.3−3 Bq, +; 1−82 Bq, ++; above 83 Bq, +++.

overview in **Table 2** is based on the conversion rates observed, which were grouped in four different categories: high activity (conversion rates above 51%), moderate activity (conversion rates from 16 to 50%), low activity (conversion rates from 4 to 15%), and no activity (conversion rates below 4%). In the case of PAL and CHS/CHI, the amount of product formed is quantified at the scintillation counter after extraction of the product with ethyl acetate while the substrate remains in the aqueous phase. For PAL and CHS/CHI activities the four groups are high activity (above 83 Bq; 95 pmol for PAL, 30 pmol for CHS/CHI), moderate activity (3.1−82 Bq; 3.5−94 pmol for PAL, 1.2−30 pmol for CHS/CHI), low activity (1.3−3 Bq; 1.5−1.9 pmol for PAL, 0.5−2 pmol for CHS/CHI), and no activity (below the threshold of 1.3 Bq (1.5 pmol for PAL, 0.5 pmol for CHS/CHI). The differences for PAL and CHS/CHI result from the different specific activities of the respective radiolabeled substrates.

For plum fruits, blackberry, gooseberry, raspberry, and sour cherry, protocol 2 was the superior method for obtaining preparations with high activities of the five target enzymes (**Table 2**). In unripe plum, however, no PAL activity could be measured with any of the protocols, although the absence of PAL in this tissue seems to be very unlikely. For elder, kiwi fruit, and plum leaves, higher activities were generally observed with protocol 1. Cherry leaves and fruits showed comparable enzyme activities with preparations from both protocols. The only exception was PAL, which could only be detected in enzyme preparations obtained from cherry fruits with protocol 2.

Our studies showed that protocol 2, which was adapted to the presence of polyphenol compounds is frequently the superior protocol for the investigations of various fruit tissues. However, the results indicate that it is important to test the protocols for each fruit species. As protocol 1, unlike protocol 2, is a simple and rapid procedure, simultaneous preparation of different samples is facilitated. Therefore, protocol 1 would be the preferred method whenever possible. Our investigations have provided the basis for future studies of eight fruit species.

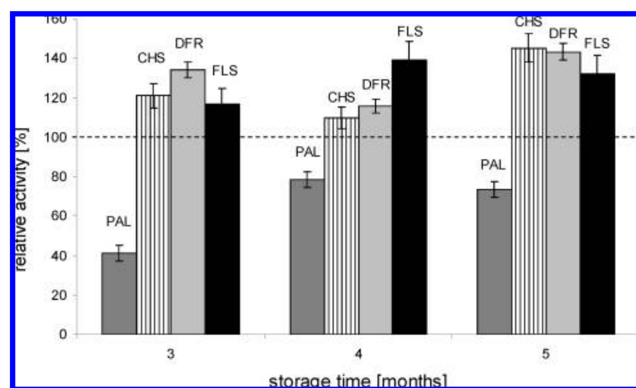

Figure 2. Relative activity [%] of selected flavonoid enzymes in kiwi fruits after different storage times in a controlled atmosphere. Values were calculated in comparison with the activities measured at harvest time. The broken line indicates the relative activity at harvest time (100% for all enzymes). At harvest time (month 0), 100% corresponds to 283 nkat/kg total protein for PAL, 272 nkat/kg total protein for CHS, 104 nkat/kg total protein for DFR, and 14 nkat/kg total protein for FLS.

Flavonoid Enzyme Activities in Ripe Kiwi Fruits. Using enzyme preparations obtained from kiwi fruits, we investigated for the first time the presence of key enzymes from the flavonoid pathway. PAL, CHS/CHI, DFR, and FLS were present with high activities, but no FHT activity could be observed. This provides an explanation for the absence of red-colored anthocyanins in the green cultivar Hayward, as is the case for most kiwi fruit cultivars, which generally have ripe fruits with yellow or green flesh (*23*). Surprisingly, kiwi fruits stored for several months still showed high flavonoid enzyme activities. The activities of CHS/CHI, FLS, and DFR were even higher compared with that at harvest time (**Figure 2**). PAL activity was lower than that at harvest but still clearly present. Thus, it is possible that the flavonoid spectrum in the fruits is modified during storage. As the flavonoid

contents and concentrations of fruits are an important aspect of quality (*24*, *25*), future studies will consider postharvest flavonoid metabolism in stored fruits and will investigate the possibility of influencing activities and the corresponding flavonoid spectrum. This could be also of relevance to the management of postharvest diseases (*26*−*29*).

ABBREVIATIONS USED

BSA, bovine serum albumine; CHS/CHI, chalcone synthase/chalcone isomerase; DFR, dihydroflavonol 4-reductase; DIECA, diethyl dithio carbamic acid; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; PAL, phenylalanine ammonia lyase; PEG, polyethylene glycol; RH, relative humidity; TLC, thin layer chromatography.

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NOTE ADDED AFTER ASAP PUBLICATION

The original web posting of May 8, 2009, contained an error in the protein content units in **Table 1**. This has been corrected in the posting of May 12, 2009.

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