Lipoxygenase and Hydroperoxide Lyase Activities in Ripening Strawberry Fruits

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The enzymes lipoxygenase and hydroperoxide lyase have been identified in strawberry (*Fragaria* × *ananassa* Duch.) var. Camarosa. Their subcellular localization, substrate preference, and product specificity were determined in mature strawberry fruits. The activity of both enzymes was located mainly in the microsomal fraction. Linolenic acid was the preferred substrate for strawberry lipoxygenase, forming 13- and 9-hydroperoxides of this acid in the proportion 70:30. The strawberry hydroperoxide lyase cleaves 13-hydroperoxide of linoleic (13% relative activity) and linolenic (100% relative activity) acids to form hexanal and (3*Z*)-hexenal, respectively. Both enzyme activities and endogenous content of volatile aldehydes formed by sequential action of lipoxygenase−hydroperoxide lyase were evaluated during strawberry development and ripening. A sequential enzymatic pathway for the formation of green odor compounds in strawberry is proposed.

Keywords: *Strawberry fruit; ripening; aroma; lipoxygenase; hydroperoxide lyase*

INTRODUCTION

Fruit aroma is a complex mixture of a large number of volatile compounds; the composition is specific to species and often to varieties. In some cases the typical fruit aroma can be ascribed to a specific compound, but in general the overall aroma quality is the sum of a multitude of components. Strawberry aroma is determined by alcohols, aldehydes, esters, sulfur compounds, and furanone-derived compounds (Dirinck et al., 1981; Pérez et al., 1992; Sanz et al., 1993a). Despite the exhaustive information regarding strawberry volatile composition, few studies have been done in relation to aroma biosynthesis on the biochemical pathways within the metabolism of the three main classes of aroma precursors: fatty acids, amino acids, and carbohydrates. In this sense, we have studied strawberry free amino acids as potential precursors of volatile alcohols (Pérez et al., 1992), and we have purified and characterized strawberry alcohol acyltransferase, the key enzyme in ester formation in fruits (Pérez et al., 1993). Preliminary work on the role of different sugars as potential precursors of Furaneol [2,5-dimethyl-4-hydroxy-3(2*H*)-furanone] biosynthesis has also been done by our research group (unpublished data). To the best of our knowledge, only the work by Schöttler and Boland (1995, 1996) on the synthesis of lactones has been published on fatty acid metabolism in relation to aroma biosynthesis in strawberry.

Fatty acids are quantitatively the major precursors of volatile compounds responsible for the aroma of plant products. Many of the alcohols, aldehydes, acids, and esters found in fruit and vegetable aromas are generated from the oxidative degradation of linoleic and linolenic acids. Aliphatic C-6 compounds, which decisively contribute to the "green odor" of fruit flavors, are formed from unsaturated aliphatic C-18 fatty acids by lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities. Hatanaka (1993) reviewed catalytic and mechanistic aspects related to this enzyme system involved in the formation of green odor in leaves. Apart from green leaves, this biosynthetic system has been demonstrated in fruits such as cucumber (Galliard and Phillips, 1976), tomato (Galliard and Matthew, 1977; Riley et al., 1996), olive (Olías et al., 1993), and bell pepper (Shibata et al., 1995). LOX gives rise to the specific hydroperoxidation of polyunsaturated fatty acids with a (*Z*,*Z*)-pentadiene structure, with oxygen incorporation occurring at C13, C9, or unspecifically at C9 and C13. This enzyme has been purified and characterized in many plant systems (Vick and Zimmerman, 1987; Sanz et al., 1997). In contrast, HPL, which cleaves these fatty acid hydroperoxides to aldehydes and oxoacids, is a membrane-bound enzyme present in small amounts in plant tissues, and it has been purified only in tea leaves (Matsui et al., 1991), bell pepper fruits (Shibata et al., 1995), and tomato leaves (Fauconnier et al., 1997). On the basis of substrate specificity, HPL proteins are divided into three types: 9-HPL, 13-HPL, and nonspecific HPL. This substrate specificity determines aroma composition of many plant products despite the specific action of LOX. In adition to their role in aroma biosynthesis, both enzymes LOX and HPL may have physiological relevance because some LOX−HPL products have antimicrobial and/or antifungal activity and are implicated in plant wounding response (Sanz et al., 1992). Because fruit tissue has both physiological and food-chemical significance distinct from other plant tissues, we thought it might be of interest to characterize LOX and HPL in strawberry and determine their activities along fruit ripening. Changes in HPL and LOX activities during fruit maturation could confirm the relationship between the enzyme activities and flavor of the fruits. The aim of this work is to contribute to a

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better knowledge of LOX and HPL physiological functions in fruits.

EXPERIMENTAL PROCEDURES

Materials. Strawberries (*Fragaria* × *ananassa*) var. Camarosa were used in this study. Strawberry plants were greenhouse grown and marked on blooming days. Fruit maturity was expressed as days after blooming (dab).

Chemicals. Dithiothreitol (DTT), polyvinylpolypirrolidone (PVPP), Triton X-100, Tween-20, linoleic acid (LA), and linolenic acid (LNA) were from Sigma Chemical Co. (St. Louis, MO). Soybean lipoxygenase was from Fluka Chemie AG (Buchs, Switzerland). (3*Z*)-Hexenal was kindly supplied by M. L. Fauconnier (Faculté Universitaire des Sciences Agronomiques in Gembloux, Belgium). The other chemicals and solvents were of analytical grade.

Fractionation of Crude Extracts. Mature red strawberries were used for this study. Strawberries were cut symmetrically into four pieces. Eight pieces of eight different strawberries (50 g) were blended in a Waring blender with 8 g of PVPP and 66 mL of 0.1 M Tris-HCl, pH 8.0, buffer. The resulting homogenate was vacuum filtered through Whatman No. 1 filter paper and the residue washed two times with 16 mL of the buffer described above. The extract was centrifuged at 12000*g* for 20 min, the pellet was discarded, and the supernatant was used as crude extract. To localize LOX and HPL proteins, a fractionation of the crude extract was carried out as follows: First, crude extract was ultracentrifuged at 250000*g* for 120 min, giving rise to a microsomal pellet. The resulting supernatant was further ultracentrifuged at 350000*g* for 120 min, and a post-microsomal pellet (PMP) and a soluble cytosolic fraction were obtained. Microsomes were washed with the extraction buffer and resuspended in three different buffers (0.1 M Tris-HCl, pH 8; 0.1 M Tris-HCl, pH 8, 1 M KCl; and 0.1 M Tris-HCl, pH 8, 0.1% Triton X-100), followed by recentrifugation at 250000*g* for 120 min.

Preparation of LOX and HPL Crude Extracts. Due to different solubility and inhibition problems of LOX and HPL enzymes, two different extraction buffers were used to prepare crude extracts of both enzymatic activities. A buffer of 0.1 M Tris-HCl, pH 8, and 1 M KCl was used for LOX extraction, and the same buffer with Triton X-100 (0.1%) was used to extract HPL activity. Strawberry tissue (25 g) was blended in a Waring blender with 4 g of PVPP and 33 mL of the appropriate extraction buffer. The resulting homogenates were vacuum filtered through Whatman No. 1 filter paper, and the residue was washed two times with 8 mL of the appropriate buffer. The extracts were centrifuged at 20000*g* for 20 min, and resulting supernatants were used as crude extracts for each enzyme.

Enzymatic Assays. *Lipoxygenase Assay.* Lipoxygenase activities were determined by continuously monitoring the formation of conjugated diene at 234 nm (Axelrod et al., 1981). The standard assay mixture consisted of 1.25 mL of 0.1 M sodium phosphate buffer, pH 6, 25 μL of substrate solution (10 mM LNA) and 25 μL of enzyme solution. One unit of LOX activity is defined as the amount of enzyme catalyzing the formation of one μmol of product/min.

Hydroperoxide-lyase Assay. Two methods for assaying HPL activity were used in this study (Olías et al., 1990a). For a rapid localization of activity in the purification process, hydroperoxide decomposition was monitored by following the decrease in absorbance at 234 nm. In this spectrophotometric assay the activity of all hydroperoxide-degrading enzymes is determined and globally expressed as lipid hydroperoxidase activity (LHP). The reaction mixture contained 1 mL of 200 mM sodium phosphate buffer, pH 6, 50 μM 13-hydroperoxylinolenic acid (13-HPOT), and the appropriate amount of enzyme (10−50 μL). Changes in absorbance at 234 nm were recorded for 60 s, and one unit of LHP activity was expressed as the amount of enzyme consuming 1 μmol of 13-HPOT in 1 min. An extinction coefficient of 25 000 M^{-1} cm^{-1} was used for 13-HPOT. The second method is based on the direct analysis of formed aldehyde by headspace gas chromatography (HS-GLC), and it is specific for HPL activity measurement. The reaction mixture described for the spectrophotometric assay was incubated at 25 °C for 15 min in an 11 mL sealed vial. The reaction was stopped by adjusting to pH 3 with 12 N HCl. The vial was then transferred into an automatic headspace sampler (Hewlett-Packard 19395A), where a 15 min equilibrium time at 80 °C was set to allow the produced aldehyde to enter the gas phase. The reaction product was determined by GLC in a Hewlett-Packard gas chromatograph (Hewlett-Packard 5890A), equipped with a FID and a glass column (1 m × 2 mm) containing 5% Carbowax 20M on 60/80 Carbopack B as stationary phase. Oven temperature was held isothermally at 120 °C. The amount of aldehyde was calculated from a calibration curve in the range 15−1500 nmol. One unit of HPL activity was defined as the enzyme forming 1 μmol of (3*Z*)-hexenal per minute.

Protein Determination. Protein was measured according to the method described by Bradford (1976), using the Pierce Coomassie protein assay reagent with crystalline BSA as the standard protein.

Determination of Endogenous C-6 Aldehydes. The endogenous level of C-6 aldehydes was determined in strawberry tissue disks. A 4 mm transverse section of the maximum diameter was cut from each fruit. From this transverse section, four disks (8 mm diameter) were obtained. Four disks from four different fruits were placed in a vial containing 1 mL of 200 mM sodium phosphate buffer previously adjusted to pH 3 with 12 N HCl. The analytical procedure for endogenous volatile measurements was the same as described above for the HPL assay.

HPL Partial Purification. All procedures were performed at 0−4 °C. Proteins with HPL activity were purified from crude extracts as follows.

Step 1. Triton X-100 was removed from the crude extract by addition of Bio-Beads SM-2 adsorbent (Bio-Rad, Hercules, CA) directly into the sample followed by stirring (180 min) and further filtration.

Step 2. $(NH_4)_2SO_4$ was added to the crude extract, and the pellet obtained at 20−75% saturation was collected.

Step 3. The $(NH_4)_2SO_4$ pellet was redisolved in 50 mM Tris-HCl buffer, pH 8, loaded onto a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column (82 × 2.8 cm), and eluted with 50 mM Tris-HCl buffer, pH 8.

Step 4. Active fractions from gel filtration chromatography were pooled, conditioned to 20 mM Tris-HCl buffer, pH 8, 0.1% Triton X-100, and 3 mM DTT and applied onto a DEAE-Sepharose fast-flow column (Pharmacia) equilibrated with the same buffer. A stepwise elution was run with the above buffer containing 0, 0.15, and 0.5 M KCl, respectively.

Analysis of LOX Reaction Products. Enzymatic reactions were carried out in the appropriate buffer using the described standard assay mixture at 20 °C with constant flow of oxygen for 15 min and then stopped by adjusting the pH to 3 with 12 N HCl. Products were extracted from the incubation mixture on reverse-phase C18 Sep-Pak cartridges (Waters, Milford, MA) and eluted with methanol. The concentrated products were esterified with diazomethane (Cohen, 1984), and the positional and geometrical isomers were analyzed by HPLC with a LiChrosorb Si-60 (250 × 4 mm, 5 μm) column (Merck, Darmstadt, Germany), eluted with *n*-hexane/diethyl ether (92:8, v/v), flow rate = 1.25 mL/min, detection at 234 nm (Sanz et al., 1993b).

HPL Substrate Synthesis and Characterization. For 13-hydroperoxylinoleic acid (13-HPOD) and 13-HPOT synthesis, 10 mg of LA or LNA, respectively, and 4 mg of commercial soybean lipoxygenase were added to 100 mL of oxygenated 0.2 M borate buffer, pH 9. The reaction was carried out at 2 °C for 15 min under a constant flow of oxygen. The 9-hydroperoxides of both polyunsaturated fatty acids (9-HPOD and 9-HPOT) were produced using LOX extracted from potato tubers (Galliard and Phillips, 1971). Hydroperoxides obtained were extracted as described in a previous section, and their purity assessed by HPLC according to the method of Sanz et al. (1993b).

RESULTS AND DISCUSSION

Subcellular Localization of Strawberry LOX and HPL. LOX and HPL were assayed in the three subcellular fractions prepared from ripe strawberry fruits according to the procedure described under Experimental Procedures. LOX activity was detected in all subcellular fractions: microsomal fraction (76% of LOX activity), cytosolic fraction (16%), and PMP fraction (7%). LOX microsomal activity could not be efficiently solubilized by increasing ionic strength (0.1 M Tris-HCl buffer, pH 8, 1 M KCl), or by using detergents (0.1 M Tris-HCl buffer, pH 8, 0.1% Triton X-10), or 0.1% Tween-20). Moreover, a reversible inhibitory effect by detergents on LOX activity was observed. When crude extracts were prepared with 0.1% Triton X-100, a very low level of LOX activity was obtained. Removal of Triton X-100 from crude extracts by means of Bio-Beads SM-2 resulted in a 9.1-fold increase in LOX activity. Crude extracts for LOX obtained with 0.1 M Tris-HCl buffer, pH 8, containing 1 M KCl exhibited high levels of LOX activity and only residual HPL activity. Strawberry LOX showed an optimum pH at 6.0, with half-maximum activity below pH 4.5 and above pH 6.7.

HPL activity was meassured by HS-GC analysis in the three subcellular fractions. Most of the HPL activity was found in the microsomal fraction (90−95%), and only 5% of activity was recovered in the cytosolic fraction. This HPL microsomal activity was not solubilized from the microsomal pellet after resuspension with 0.1 M Tris-HCl buffer, pH 8, and 1 M KCl and recentrifugation at 250000*g* for 120 min. HPL activity was efficiently extracted (100%) when resuspension was done in 0.1 M Tris, pH 8, and 0.1%Triton X-100. This fact demonstrates that strawberry fruit HPL is a membrane-bound enzyme. Thus, to obtain good levels of HPL activity in crude extracts, 0.1% Triton X-100 was used in the extraction buffer. Gardner et al. (1991) showed that HPL activities of soybean seeds, seedlings, and leaves required solubilization by detergent for in vitro maximum activity, and proteins with HPL activity identified in pear, tomato, and bell pepper fruits were also described as membrane proteins (Kim and Grosch, 1981; Shibata et al., 1995; Riley et al., 1996).

HPL Partial Purification. Proteins with HPL activity in crude extracts were precipitated by ammonium sulfate fractionation. Preliminary experiments indicated the need of removing Triton X-100 to obtain clear protein precipitates. Detergent (0.1% Triton X-100) was removed by addition of Bio-Beads SM-2 adsorbent directly into the sample followed by stirring (180 min) and further filtration. Precipitated proteins between 20 and 75% ammonium sulfate saturation were applied onto a Sephacryl S-300 column, where an early single peak of HPL activity was determined. Fractions containing HPL activity were combined, conditioned to 20 mM Tris-HCl buffer, pH 8, 0.1% Triton X-100, and 3 mM DTT, and loaded onto a DEAE-Sepharose fast-flow column equilibrated with the same buffer. Addition of Triton X-100 (0.1%) and DTT (3 mM) proved to be essential for the elution of HPL protein, similarly to what was found in tomato leaf HPL (Fauconnier et al., 1997). A stepwise elution was carried out, and the HPL activity was eluted at 0.15 M KCl in the equilibrium buffer. The HPL specific activity of this enzymatic extract was 15.6 U/mg of protein, with a purification factor of 21-fold and a recovery of 1.5%. HLP activity was routinely assayed by the spectrophotometric method through the purification process, and the HS-GC analysis was used to assess the HPL specific activity at each purification step. The decrease in absorbance at 234 nm accounts for all hydroperoxide-degrading enzymes (LHP), whereas the HS-GC analysis is based on the direct analysis of aldehydes specifically formed by HPL. In crude extract HPL activity values (HS-GC analysis) were only 75% of total LHP activity (decrease in absorbance at 234 nm), whereas in the DEAE pool 100% of the LHP was identified as HPL.

Table 1. Substrate Specificity of Strawberry LOX

substrate	LOX activity (mU/g of FW)	relative activity (%)
LA	613.78[a]	55.6
LNA	1103.91	100
Y-LNA	500.49	45.34
ARA	161.83	14.68

[a] Values are the average of six determinations.

HPL Properties. The partially purified enzyme preparation eluted from DEAE-Sepharose was used to determine the optimum pH for HPL. HPL exhibited a maximum activity at pH 6, with 40% activity below pH 4.5 and above pH 8. Optimum pH values described for HPL range from 5.5 in green bell pepper fruits (Shibata et al., 1995) to pH 8 in cucumber seedlings, with most described HPL proteins working at neutral pH (Kim and Grosch, 1981; Matsui et al., 1991; Olías et al., 1990b). To study substrate specificity, the purified enzyme was incubated with isomeric 9- and 13-HPOD and 9- and 13-HPOT. Strawberry HPL showed no activity with the 9-isomers and was active against the 13-hydroperoxides. Relative activity values of strawberry HPL against 13-HPOT and 13-HPOD were 100 and 13%, respectively. These results are in good agreement with substrate preference determined for strawberry LOX, with maximum activity with LNA as substrate (Table 1). Analysis of reaction products of strawberry LOX with LNA as substrate indicated that 13-HPOT and 9-HPOT were formed in a ratio 70:30. Similar specificity has been found for HPL from tea leaves (Matsui et al., 1991), tomato fruits (Galliard and Matthew, 1977; Galliard et al., 1977), tomato leaves (Fauconnier et al., 1997), olives (Olías et al., 1993), and bell pepper fruits (Shibata et al., 1995). Different results were described for the enzymes from pear and cucumber fruits that cleave exclusively the 9-hydroperoxides of LA and LNA (Kim and Grosch, 1981; Galliard and Phillips, 1976). Kinetic studies were carried out with partially purified strawberry HPL enzyme and two different substrates, 13-HPOT and 13-HPOD. The apparent K_m value for strawberry HPL with 13-HPOT was estimated from a Lineweaver−Burk plot as 62 μM and a relative V_{max} = 4.3 mM/min. When 13-HPOD was used as substrate, the kinetic values were K_m = 102 μM and V_{max} = 0.3 mM/min. These K_m and V_{max} values show a greater affinity of strawberry HPL for 13-HPOT. Kinetic values found in this study were quite similar to those calculated for tomato leaf HPL (Fauconnier et al., 1997) but higher than those found for bell pepper HPL (Shibata et al., 1995).

Evolution of LOX and HPL during Strawberry Ripening. LOX and HPL activities were followed along strawberry development and ripening, from green small fruits (14 dab, approximate weight of 1 g/fruit) to dark red overripe fruits (37 dab, approximate weight of 27 g/fruit). Five fruit maturity stages were established according to external color: I, green; II, white; III, pink;

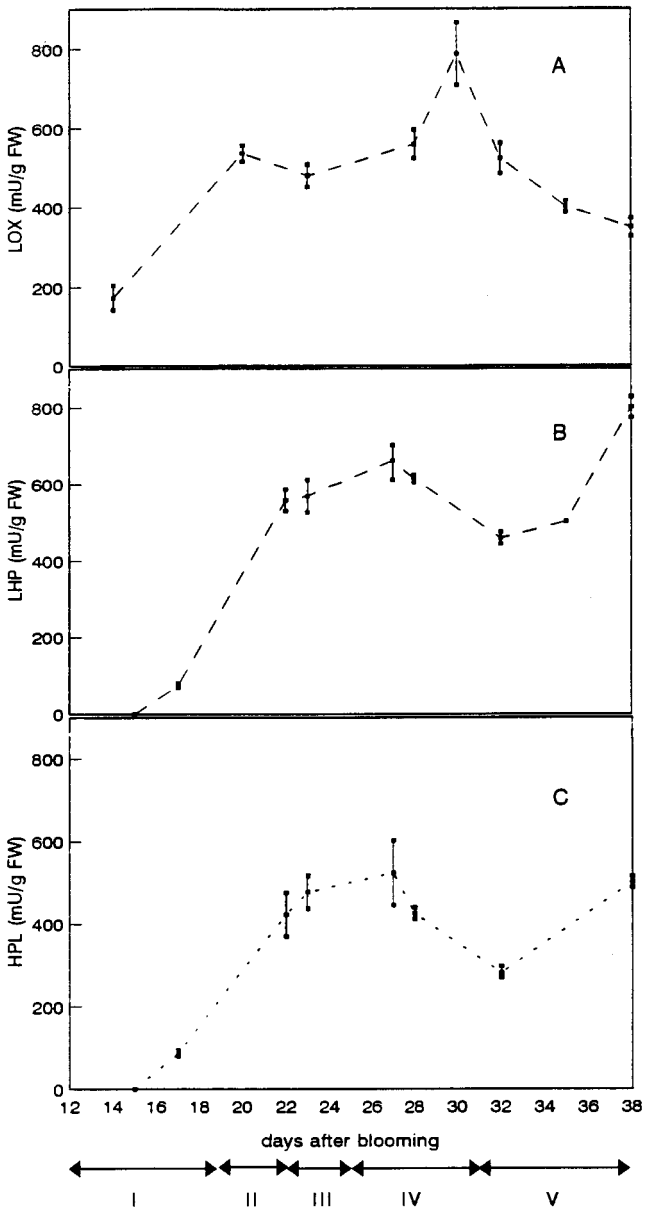

Figure 1. LOX (A), LHP (B), and HPL (C) activity profiles during strawberry ripening. LNA was used as substrate for LOX activity, and 13-HPOT was used for LHP and HPL activities. Visual maturity stages are shown at the bottom. Each value is the average of four determinations. Vertical bars indicate ±SD.

IV, bright red; and V, dark red fruits (Figure 1). Comparison of HPL activity (Figure 1C) to LPH activity (Figure 1B) indicated that HPL is the main hydroperoxide-metabolizing enzyme in all stages, accounting for 65−80% of the hydroperoxide metabolization. The increase in LOX (Figure 1A) and HPL activities takes place at the same developmental stage, stage II, ~20 dab, suggesting that the expression of both proteins was cooperatively regulated, similarly to what Matsui et al. (1997) postulated for LOX and HPL in bell pepper fruits. A steady increase was observed in both enzymatic activities from stage II to stage IV. During the period of time examined, LOX and HPL had quite similar values of catalytic activity. LOX activity profiles showed a sharper profile with maximum activity at 30 dab (stage IV), whereas HPL activity exhibited a broader range of activity at earlier maturity stages. Differences in the behavior of both enzyme activities were found in

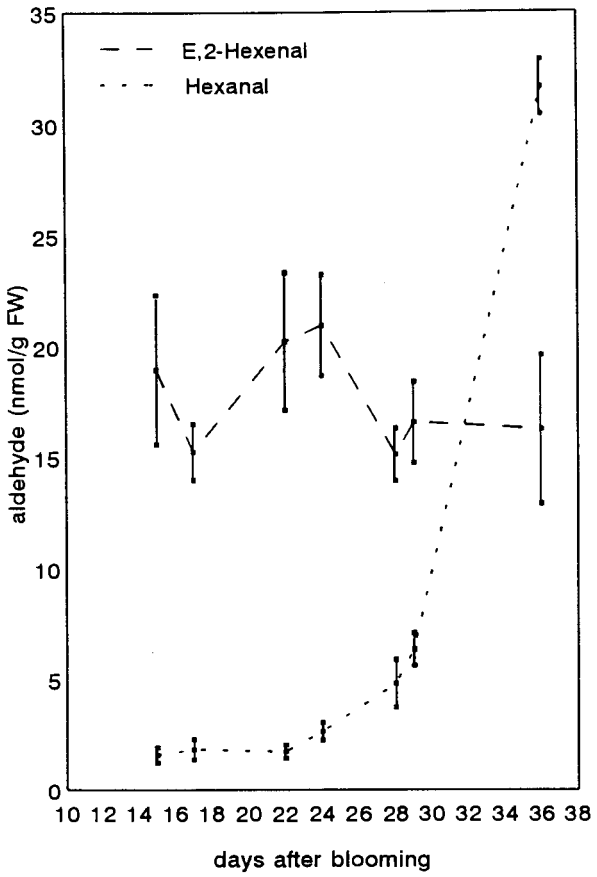

Figure 2. Endogenous C-6 aldehydes content during strawberry ripening. Each value is the average of four determinations. Vertical bars indicate ±SD.

overripe fruits in which a decrease in LOX was concomitant with an increase in HPL activity. The higher HPL activity could be explained as a response to increased available substrates caused by membrane disorganization and increased lipid peroxidation associated with fruit senescence. In this sense, with similar levels of LOX activity the increase of LOX substrates would cause a higher endogenous level of fatty acid hydroperoxides (highly cytotoxic) that would trigger all hydroperoxide-metabolizing enzymes as reflected in Figure 1, with a maximum HPL value for 38 dab fruits.

The C-6 aldehydes formed by the sequential catalytic activity of LOX−HPL were also analyzed during strawberry ripening (Figure 2). (2*E*)-Hexenal was the major endogenous aldehyde found in most developmental stages, with only a trace amount of (3*Z*)-hexenal. In most plant products the compounds having a (3*Z*)-enal structure are quickly isomerized by an isomerase enzyme to the (2*E*)-enal form (Phillips et al., 1979). The formation of a greater amount of unsaturated C-6 compounds than of saturated ones could indicate that the in vivo substrate for LOX-HPL was LNA and that substrate and product specificity found in the in vitro assay could reflect the in vivo specificity of both enzymes. Endogenous (2*E*)-hexenal contents do not change significantly during strawberry ripening. By contrast, a sharp increase in hexanal production in overripe strawberries was observed (Figure 2). This hexanal overproduction could be attributable to a change in HPL specificity along fruit ripening, as proposed by Matsui et al. (1997). These C-6 aldehydes are not only present as green odor notes in strawberry flavor but also act as precursors in the biosynthesis of hexyl and hexenyl

esters. In previous studies we purified and characterized the strawberry ester-forming enzyme, alcohol acyltransferase (AAT), with marked specificity for C-6 alcohols (Pérez et al., 1993), and we followed strawberry AAT activity during fruit development and ripening, showing a maximum AAT activity in fully mature red fruits (Pérez et al., 1996). When the HPL activity profile is compared with that of AAT, the higher HPL activity found in pre-mature strawberry fruits gives good evidence of a sequential enzymatic pathway for the formation of green odor compounds in strawberry.

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