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Mini Review Article

Oxylipin Pathway in the Biosynthesis of Fresh Tomato Volatiles

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Abstract : Fresh tomato volatiles are formed in intact fruit during ripening and upon tissue disruption. There are different pathways involved in the biosynthesis of these volatiles. The oxylipin pathway uses free unsaturated fatty acids with the sequential action of lipoxygenase, hydroperoxide lyase and alcohol dehydrogenase to produce volatile aldehyde and alcohol compounds. Oxylipin volatiles are the most important components in fresh tomato aroma. In order to genetically improve the quality of tomato aroma, the biochemistry of aroma synthesis must be studied thoroughly. This review examines current knowledge of the subject and opens new areas for further investigation.

Key Words: Tomato, lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase, volatile, synthesis

Taze Domates Volatilleri Biyosentezinde Oksilipin Reaksiyonları

Özet : Taze domates volatilleri bütün meyvenin olgunlaşması ve dokunun zedelenmesi sonucu oluşur. Volatillerin biyosentezinde çeşitli reaksiyonlar rol alır. Oksilipin reaksiyonları doymamış serbest yağ asitlerini kullanarak, lipoksigenaz, hidroperoksit liaz, ve alkol dehidrogenazın sırayla reaksiyonları sonucu volatil aldehit ve alkol bileşenlerini üretirler. Oksilipin volatilleri taze domates aromasının en önemli bileşenleridir. Domates aroma kalitesinin genetik yollarla artırılabilmesi için, aroma sentezinin biyokimyası detaylı olarak çalışılmalıdır. Bu derleme şimdiki bilgileri toplar ve ileri araştırmalar için yeni alanlar açar.

Anahtar Sözcükler: Domates, lipoksigenaz, hidroperoksit liaz, alkol dehidrogenaz, volatil, sentez

Introduction

Flavor and aroma are essential parameters of quality in fresh tomatoes (1). Flavor volatiles are formed in the intact tomato fruit during ripening as well as upon tissue disruption, which occurs when tomatoes are macerated, blended, or homogenized (2). Tomato volatiles have been divided into six groups: a) lipid derived, b) carotenoid related, c) amino acid related, d) terpenoids, e) lignin related, and f) other misceleneous (3). This division implies different pathways, substrates, and enzymes for the biosynthesis of the aroma volatiles. It is very important to study the biochemistry of aroma biogeneration in fresh tomato, since moleculer

biologists and plant breeders do not have clear targets for the genetic manipulation of flavor (4).

Oxylipin volatiles are considered by far the most important components of tomato aroma, but the exact roles in perception and synthesis patterns require further investigation.

The Oxylipin (Lipoxygenase) Pathway

It has been thought that in plant tissues lipid peroxidation occurs through enzymatic processes, such as those of the oxylipin / lipoxygenase (LOX) pathway (5). The products of lipoxygenase pathway reactions depend upon the nature of the cellular stimulus, the available substrates, and the intracellular site of the reactions. Oxylipin is the generic name for a family of oxygenated compounds formed from fatty acids by enzymatic reactions. They include fatty acid hydroperoxides, hydroxy fatty acids, epoxy fatty acids, keto fatty acids, volatile aldehydes, and cyclic compounds. The oxylipin pathway influences aroma, taste, and possibly the deterioration of fresh plant products (5, 6). Although some lipoxygenase isoenzymes can oxidize specific glyceride lipids, it is generally acknowledged that free polyunsaturated fatty acids are the preferred substrates. General substrates for lipoxygenase are fatty acids containing a cis, cis-1,4-pentadiene structure. The main substrates for lipoxygenase in plants are linoleic and linolenic acid, while in mammals these are arachidonic and eicosapentaenoic acid (5, 6, 7).

There are several branches of the pathway in plants which produce important plant growth regulators, like jasmonic acid and plant hormone traumatine. The main concern of this review is the generation of volatile aldehydes. Upon lipolysis, the first enzyme in the series is LOX. Lipoxygenase catalysis consists of three stages: (a) stereospecific hydrogen removal from the methylene group between two double bonds; (b) antarafacial allylic rearrangement of the resulting free radical; and (c) binding of molecular oxygen to the 2-trans,4-cis-pentadienyl-1 radical. Stage one is considered to be the rate-limiting catalysis. All presently known plant LOXs attack the prochiral center C-11 of linoleic and linolenic acids. The resulting bis-allylic radical undergoes either (n+2) or (n-2) isomerization into allylic radical. Most LOX enzymes exhibit high regiospecificity. They transform linoleic acid into 13(S)-hydroperoxy-cis-9,trans-11-octadecanoic acid (13S-HPOD) and 9(S-hydroperoxy-trans-10,cis-12-octadecanoic acid (9S-HPOD), and transform linolenic acid into 13(S)-hydroperoxy-cis-9,trans-11,cis-15-octadecatrienoic acid (13S-HPOT) and 9(S)-hydroperoxy-trans-10,cis-12,cis-15-octadecatrienoic acid (9S-HPOT) (6, 7, 8).

The above-mentioned hydroperoxides then serve as substrates for several pathways: the hydroperoxide lyase pathway (HPLS), the hydroperoxide dehydratase pathway (HPDS), the hydroperoxide isomerase pathway (HPIS) (or allene oxide synthase pathway), and the hydroperoxide-dependent peroxygenase (HPPR) / epoxygenase (HPEP) pathway (5).

There is both 'heterolytic' and 'homolytic' hydroperoxide lyase metabolism of the fatty acid hydroperoxides. The homolytic path is anaerobic and cleavage occurs at the free-radical level. Heterolytic hydroperoxide catalyzes the chain cleavage of hydroperoxide fatty acids between the hydroperoxide carbon and the neighboring double methine group. By means of this reaction, C6 aldehydes and a C12 aldoacid are produced from 13-hydroperoxides, and C9 aldehydes and a C9 aldoacid are produced from 9-hydroperoxides. Figure 1 shows these reaction paths and products (4).

The mechanism of hydroperoxide lyase (HPL) action involves: (a) protonation-dehydration of the hydroperoxide, which leads to epoxyallylic carbocation; (b) rearrangement of the epoxyallylic cation into oxonium ion; (c) hydroxylation of oxonium ion into hemiacetal; and (d) decomposition of hemiacetal into two aldehyde fragments. The primary products of the HPL reaction are important components of the characteristic aromas of many fruits and vegetables (6, 8).

Primary HPL products are further converted by: (a) allylic isomerization of 3-cis-alkenals into 2-trans-alkenals, i.e., 3-cis-hexenal into 2-trans-hexenal (this reaction may be spontaneous or may occur through the action of alkenal isomerases); (b) aldehyde reduction by alcohol dehydrogenase (ADH) presenting 3-cis -hexenal reduction to 3-cis-hexenol; and (c) oxygenation

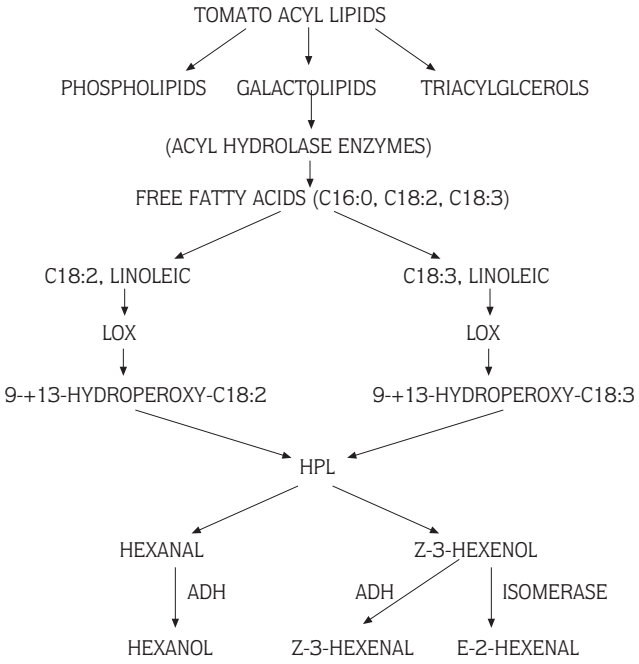

Figure 1. Formation of lipid-derived volatiles through LOX pathway (4).

of 3-cis-alkenals into corresponding hydroperoxides and hydroxy derivatives (8). The products of these reactions have been shown to play physiological roles in such actions as wound healing, cystostatic activity, DNA damage, non-specific covalent protein modifications, antimicrobial activity, pest resistance, fungitoxicity, senescence, secondary metabolite induction, ethylene stimulation, growth inhibition, and aroma formation (5, 6, 7, 8).

Tomato Lipoxygenase

The term lipoxygenase (LOX, EC 1.13.11.12) (linoleate:oxygen oxidoreductase) refers to a group of enzymes that catalyze dioxygenation, by use of molecular oxygen, of methylene-interrupted polyenoic fatty acids into corresponding enantiomeric hydroperoxy fatty acids. Multiple forms or isozymes exist in many tissues, showing differences in regiospecificity, optimum pH, pI and enzymatic properties. Most are composed of a single polypeptide chain of approximately 850 amino acids with an average molecular weight of 96 kDa. They contain one mol of non-heme iron per mol of enzyme. The inactive form is E-(Fe+2), and it is activated by reaction products to the oxidized E-(Fe+3) form. Because of this product activation requirement, it exhibits a characteristic initial lag period. LOXs are inactivated by its substrate analogs or by lipid antioxidants i.e., α-tocopherol, nordihydroguaiaretic acid (NDGA), propyl gallate, hydroquinone, and α-naphthol. LOX is also a self-destructing enzyme, and the velocity of catalysis decreases linearly with substrate utilization (5, 8, 9).

Tomato lipoxygenases have been widely studied. Apparent molecular weights of 87 to 95 kDa have been reported (10, 11, 12). Optimum pH can range from 4.2 to 8.0, and pI values of 4.2 and 5.1 have been reported. LOXs in the microsomal fraction show a wider range (10, 11, 13, 14). Although one study reported a linear increase in LOX activity by fruit ripening (15), others reported that the highest level of activity was at the breaker stage, subsequently decreasing through the full ripe stage (10, 14). Greater amounts of LOX mRNA and protein have been found in the breaker to ripe and red-ripe stages (16). LOX activity in tomato fruits has been reported both in membranous systems (11, 13, 14) and in soluble form (10, 12, 14). The highest level of LOX activity was found to exist between the skin and outer flesh of the fruit (17). However, the greatest amount of LOX mRNA and protein was found to be in the locular jelly and radial walls of ripe fruit (16). Enzyme activity has been assayed by colorimetric, manometric, polarographic, spectrophotometric and radiolabeled substrate monitoring assays (11, 12, 13, 14, 18). The values of Km and Vmax vary due to purity and assay differences. In one study, a Km of 4.1 mM and a Vmax of 7.4 mM min-1 mg-1 protein were determined (12), while in another study an apparent Km of 0.52 mM and a Vmax of 0.186 mM min-1 mg-1 protein (13) were reported. Strong inhibition of tomato lipoxygenase by nordihydroguaiaretic acid (10), n-propyl gallate (13), and a fungal glucose oxidase (19) has also been shown. UV irradiation of tomato fruit has also been found to cause increases in the formation of hexanal (17). It has been suggested that LOX activity is latent and begins to express only upon injury (17). Product analysis has revealed a 96 % product specificity of 9-HPOD formation from

linoleic acid with an 82 % S enantiomeric excess. There was 1% formation of the other product, 13-HPOD, and this was found to be racemic (12).

Tomato Hydroperoxide Lyase

Another important enzyme in the pathway is hydroperoxide lyase (HPL), which is not listed in enzyme classifications (8). The molecular weight of this enzyme is in the range of 200-250 kDa and it is thought to be a tetramer of 62 kDa each (5, 8), but tomato leaf HPL is shown to be a trimer of 73 kDa each to a molecular mass of 216 kDa (20). The enzyme is located in chloroplasts and non-chloroplastic particles (21), and appears in both membrane-bound and soluble forms (8). It exhibits no tissue specificity (17). Solubilization by Triton X-100 from tomato leaf supports membrane association (20), and 73 % of total activity at all stages of tomato maturity has been shown to occur in the microsomal fraction. The activity of the enzyme does not change during tomato fruit ripening (14). The location of HPL normally corresponds to the location of LOX found in the same tissue (8). There are three classes of HPLs for substrate specificity: (a) 9-hydroperoxide specific HPL produces C9 aldehydes and C9 oxoacids; (b) 13-hydroperoxide specific HPL yields C6 aldehydes and C12 oxoacid; and (c) nonspecific PPL acts on both substrates. There is also a 10-hydroperoxide-specific HPL found in mushrooms which produces an alcohol, 1-octen-3-ol (21). Tomato HPL is 13-hydroperoxide specific (20, 21), despite the fact that tomato LOX is 9-hydroperoxide specific; thus, the availability of substrates can be the rate-limiting factor (14). Preference for the S configuration and inhibition by p-chloromercuribenzoate has been demonstrated (21). An effective pH range was shown to be 5.5 to 8.0, while it was determined that the pI for tomato leaf HPL was 4.9 (14, 20, 21). Spectroscopic studies have demonstrated that HPL is a heme protein, specifically a heme b (protoheme IX) (5, 22). A common but time-consuming method of assaying its activity employs GC for headspace analysis of volatiles formed. Another assay is based on derivatization of the aldehyde products formed with 2,4-dinitrophenylhydrazine, followed by chromatographic and spectrophotometric measurements. A convenient but less specific assay measures spectrophotometrically at 234 nm the decrease in absorbance caused by the loss of conjugated dienes (21, 23). A specific assay for HPL is based on yeast alcohol dehydrogenase utilization of HPL products coupled with NADH, and the decrease in absorbance is measured at 340 nm. This method has proved to be highly specific, reproducible and accurate (14, 23).

Tomato Alcohol Dehydrogenase

It has also been suggested that alcohol dehydrogenase (ADH, EC 1.1.1.1) is an important enzyme in tomato flavor development (4). Although one study (24) indicated both the presence of 4 isozymes in the mature green stage, and the presence of 2 isozymes in the small-green and ripe stages of tomatoes, in another study (25), no activity was observed in green tomatoes. The highest level of ADH activity in tomatoes was observed in fully red fruit, and this may be responsible for the change of ‘fresh’ flavor notes to those described as ‘processed’ or ‘enzymic’ notes. Tomato-fruit ADH is a dimer with a molecular mass of 90-100 kDa. Atomic absorption

has proved the presence of 15-48 zinc atoms per enzyme dimer. Its coenzyme is NAD^+, and the spectrophotometric assay is based on absorbance measurement at 340 nm due to NAD^+ reduction to define a unit as the formation of 1 mmol NADH per minute. The enzyme exhibited no $NADPH^+$-dependent activity. Ethanol and acetaldehyde are the best substrates among alcohols and aldehydes, with the lowest Km and highest V values. Interestingly, the enzyme was also active on geraniol, while no activity was observed on cyclohexanal, 2-octanol, or β-ionone. Acetamide, iodoacetamide, some aromatics, 4,7-diphenanthroline, pyrazole and 2-octanol have been shown to inhibit the enzyme (2, 4, 25).

Oxylipin Volatiles in Fresh Tomato

The most important lipid-derived tomato volatiles are identified by GC-MS techniques, by holding for 3 min after blending of the tissue. They are listed in Table 1 (3).

Several studies link the existence of lipoxygenase pathway enzymes and reactions to known aroma volatiles in tomato. In one study, linoleic acid was incubated (26) with tomato homogenate and a 69 % yield of linoleic acid hydroperoxides with a 96:4 ratio of 9- to 13-hydroperoxide isomers was observed. Another study produced hexanal from tissue slices and cell-free extracts of tomato (27) under optimal conditions, a pH of 7.5 and temperature of 30 oC for 4 h. Boiling of the extract and addition of H2O2 have been found to inhibit production, whereas the addition of FeSO4 and CuCl2 have been found to increase it. Later studies have tried to identify enzyme involvement in the synthesis of flavor volatiles. Changes in major lipid

Table 1. Oxylipin volatiles in fresh tomato fruit (3).

Compound	Concentration (ppb)
1-Penten-3-one	450
1-Penten-3-ol	100
Pentanal	5
(Z) and (E)-Pentenal	100
Pentanol	30
(Z)-2-Pentenol	40
(Z)-3-Hexenal	15000
Hexanal	2000
(E)-2-Hexenal	470
(Z)-3-Hexenol	120
Hexanol	4
(E,Z) and (E,E)-2,4-Hexadienal	10
Unknown C6H8O2	74
(E)-2-Heptenal	40
1-Octen-3-one	5
(E,Z) and (E,E)-2,4-Heptadienal	5
4,5-epoxy-(E)-2-Heptenal	4
(E)-2-Octenal	15
(E,Z) and (E,E)-2,4-Decadienal	10
4,5-epoxy-(E)-2-Decenal	30

classes and free fatty acids, and the formation of C6 aldehydes and w-oxoacids have been monitored (28), and it has been suggested that there is involvement of LOX / HPL in the pathway. The effect of blending the tissue on volatile formations has also been studied. Blended and vacuum distilled samples yielded cis-3-hexenal, whereas blended and steam-distilled samples produced trans-2-hexenal. Isomerization was thought to be the reason for this (29). It was also shown that aromatic aldehydes were converted to alcohols, but the reverse was not observed. It has been suggested that the higher the linoleic/linolenic acid ratio, the higher the hexanal/hexenal ratio. Moreover, in general, the greater the amount of acyl, the greater the amount of C6 volatiles (30). When the yeast Δ-9 desaturase gene was expressed in tomato, increases in palmitoleic (16:1), oleic (18:1) and linoleic (18:2) acid levels were observed with an overall doubling of total fatty acids. Increased levels of lipid-derived volatiles: cis-3-hexenal, trans-2-hexenal, cis-3-hexen-1-ol, hexanal, trans-2-octanal, 2-nonenal, and heptanal were also noted. Levels of 6-methyl-5-hepten-2-one, which is derived from lycopene and 2-isobutylthiazole, which comes from leucine, also increased in transgenic tomato fruit (31). In a recent study designed to determine subcellular localization of major lipid-derived volatiles (32), almost 90 % of the total hexanal and trans-2-hexenol were observed in the cytosol, whereas microsomal membranes and lipid particles contained 6.5 % and 4.3 % of activity, respectively. In the microsomal fraction, the level of hexenal was 18 times greater than that of trans-2-hexenal, whereas, in cytosol and lipid particles, the level was only 4 times greater. These results suggest that lipid particles, known as deteriosomes, served as vehicles of volatile transport from membranes to aqueous cytosolic compartments. There are no current studies on the effects of cloning LOX and HPL genes on the volatile compounds produced so far. In one study, ADH activity was modified by genetic manipulation, and hexanol and cis-3-hexenol levels were increased in fruits with increased ADH activity and reduced in fruit with low ADH activity (33). In another study, the ratio of cis-3-hexenal:cis-3-hexenol and 3-methylbutanal:3-methylbutanol were altered in down-regulated fruit and not increased in up-regulated fruits (34). Fruits with enhanced ADH activity were identified as being 'riper' and 'more liked' than the controls (33).

Products of the LOX pathway are currently considered the most important components of tomato aroma volatiles, but more studies of biosynthesis and genetic manipulation are needed.

Conclusion

Lipid oxidation products by the LOX / HPL / ADH path are usually characterized by green, fresh, and grassy notes. These reactions are dominant when tissue is disrupted, upon which the substrates and enzymes are combined in the presence of oxygen. Lipid-derived volatiles occur in intact tissue during fruit ripening, although the concentration of compounds may be much smaller than in macerated tissue. The level of enzyme activity present is certainly a factor in the

biogenesis of aroma volatiles. The amounts of substrates available and the sub-cellular conditions are other factors which affect the production of volatile compounds. In addition, there are competing pathways that share the substrates and / or products.

There have been very few genetic studies that have attempted to modify the aroma quality of fresh tomatoes with oxylipin-pathway enzymes. There have been no reported studies of genetic manipulation of LOX and HPL activities to enhance volatile production . This field requires further investigation. It is also essential that the desirability of each volatile be determined by sensory evaluation and consumer tests before a target genetic transformation is selected. A single pathway and / or enzyme may produce a range of volatiles that have very different sensory notes. Individual as well as complex interactions with other volatiles and non-volatile constituents of flavor must be thoroughly evaluated. More biochemical, genetic, chemical and sensory studies are needed to improve the quality of one of the most important fresh food products, fresh market tomato, for the benefit of the health- and quality-conscious modern consumer.

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