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6

Inhibition of Soybean Lipoxygenases – Structural and Activity Models for the Lipoxygenase Isoenzymes Family

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1. Introduction

Lipoxygenases (EC 1.13.11.12, linoleate:oxygen, oxidoreductases, LOXs) which are widely found in plants, fungi, and animals, are a large monomeric protein family with non-heme, non-sulphur, iron cofactor containing dioxygenases that catalyze the oxidation of polyunsaturated fatty acids (PUFA) as substrate with at least one 1*Z*, 4*Z*-pentadiene moiety such as linoleic, linolenic and arachidonic acid to yield hydroperoxides (Gardner, 1991).

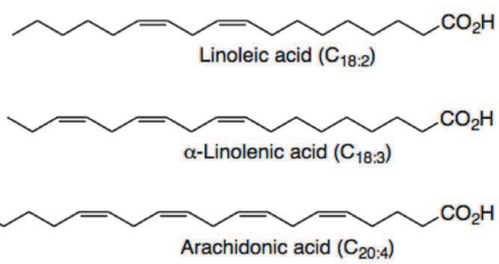


Fig. 1. Lipoxygenase substrates, linoleic, α- linolenic and arachidonic acid.

Theorell et al. (1947) succeeded in crystallizing and characterizing lipoxygenase (LOX) from soybeans and since then among plant LOXs, soybean lipoxygenase isozyme 1 (LOX-1) can be regarded as the mechanistic paradigm for these nonheme iron dioxygenases (Coffa et al., 2005; Minor et al., 1996; Fiorucci et al., 2008).
Designing agents to modulate activities of the variety of so closely homologous enzymes, such as different LOXs, require an intimate knowledge of their 3D structures, as well as information about metabolism of the potential xeno- or endobiotics. So far only the structures of soybean isozymes LOX-1 and LOX-3 have been determined for native enzymes, and several structures of their and rabbit 15-LOX (from reticulocytes) molecular complexes with inhibitors are known. Due to lack of sufficiently purified human enzymes most of the structural research has been done on soybean LOX (Skrzypczak-Jankun et al., 2003).

Understanding the mechanism of inhibition of LOXs can have profound effect in the development of many anti-cancer and anti-inflammatory drugs. On the basis of the available LOX data it was suggested that a combination of LOX modulators might be needed to shift the balance of LOX activities from procarcinogenic to anticancerogenic as a novel strategy for cancer chemoprevention (Skrzypczak-Jankun et al., 2003).
The aim of the present study is to present knowledge on different lipoxygenases having the soybean lipoxygenases as a structural and activity template for their inhibition by natural antioxidant compounds as theoretical approach for food biochemistry and medical applications.

2. Lipoxygenase structure and activity

The three-dimensional structure of soybean lipoxygenase-1 has been determined to 2.3 Å resolution by single crystal X-ray diffraction methods (Boyington et al., 1993). It is a two-domain, single-chain prolate ellipsoid of dimensions 90 x 65 x 60 Å with a molecular mass of 95 kDa. The 839 residues are organised in two domains: one 146 residue N-terminal domain (domain I), and a major, 693 residue C-terminal domain (domain II) (Prigge et al., 1997). Overall, the three-dimensional structure of lipoxygenase-1 shows a helical content of 38.0% and a β-sheet content of 13.9%. The structure of another crystal form of soybean lipoxygenase-1 determined to 1.4 Å resolution (Minor et al., 1996) showed very similar results. The structure of lipoxygenase-3, another soybean lipoxygenase isozyme (Skrzypczak-Jankun, 1997) shows that the lipoxygenase-3 isozyme is very similar in structure despite significant differences in sequence: 857 residues vs 839, deletions at 7 positions, insertions at 25 positions, and substitutions at 224 residues (72% identity).
Soybean seed isoenzymes are 94–97 kDa monomeric proteins with distinct isoelectric points ranging from about 5.7 to 6.4, and can be distinguished by optimum pH, substrate specificity, product formation and stability (Siedow, 1991; Mack et al., 1987). LOX-1 is the smallest in size (838 amino acids; 94 kDa), exhibits maximal activity at pH 9.0 and converts linoleic acid preferentially into the 13-hydroperoxide derivative. LOX-2 is characterized by a larger size (865 amino acids; 97 kDa), by a peak of activity at pH 6.8, and forms equal amounts of the 13-and 9-hydroperoxide compounds (Loiseau et al., 2001). LOX-2 oxygenates the esterified unsaturated fatty acid moieties in membranes in contrast to LOX-1 which only uses free fatty acids as substrates (Maccarrone et al., 1994). LOX-3 (857 amino acids; 96.5 kDa) exhibits its maximal activity over a broad pH range centred around pH 7.0 and displays a moderate preference for producing a 9-hydroperoxide product. It is the most active isoenzyme with respect to both carotenoid cooxidation and production of oxodienoic acids (Ramadoss, 1978).

3. Lipoxygenase reaction

The initial step of LOX reaction is removal of a hydrogen atom from a methylene unit between double bonds in substrate fatty acids (Fig. 2A). The resulting carbon radical is stabilized by electron delocalization through the double bonds. Then, a molecular oxygen is added to the carbon atom at +2 or –2 position from the original radical carbon, forming a peroxy radical as well as a conjugated *trans*,*cis*-diene chromophore. The peroxy radical is then hydrogenated to form a hydroperoxide. The initial hydrogen removal and the following oxygen addition occur in opposite (or antarafacial) sides related to the plane

formed by the 1Z,4Z-pentadiene unit. In most LOX reactions, particularly those in plants, the resulting hydroperoxy groups are in *S*-configuration, while one mammalian LOX and some marine invertebrate LOXs produce *R*-hydroperoxides. Even in the reactions of such "*R*-LOXs", the antarafacial rule of hydrogen removal and oxygen addition is conserved.
In cases of plant LOXs, including soybean LOXs, the usual substrates are C18-polyunsaturated fatty acids (linoleic and α-linolenic acids), and the products are their 9*S*- or 13*S*-hydroperoxides (Fig. 2B). Most plant LOXs react with either one of the regio-specificity, while some with both. Therefore, based on the regio-specificty, plant LOXs are classified into 9-LOXs, 13-LOXs, or 9/13-LOXs.

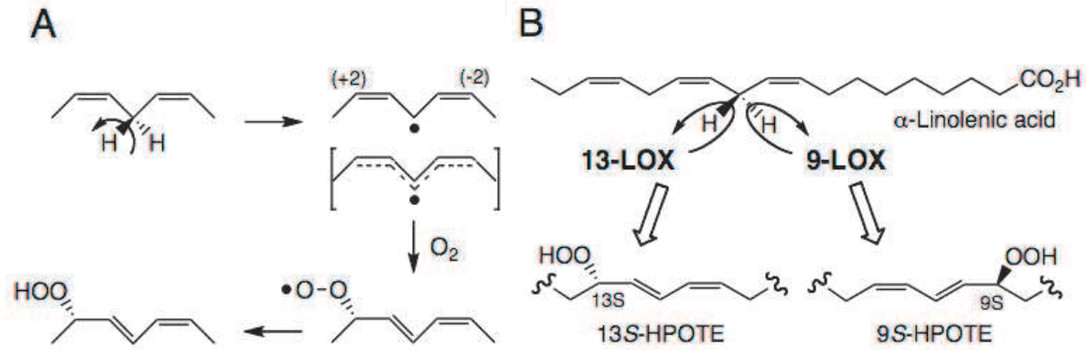


Fig. 2. LOX reaction showing the principal steps of LOX reaction (Panel A), and the actual reactions of plant LOXs and α-linolenic acid (Panel B). HPOTE: hydroperoxyoctadecatrienoic acid.

4. Biological and metabolic functions

4.1 In plants

Lipid peroxidation is common to all biological systems, both appearing in developmentally and environmentally regulated processes of plants (Feussner & Wasternack, 2002). The hydroperoxy polyunsaturated fatty acids, synthesized by the action of various highly specialised forms of lipoxygenases, are substrates of at least seven different enzyme families (Feussner & Wasternack, 2002). Signaling compounds such as jasmonates, antimicrobial and antifungal compounds such as leaf aldehydes or divinyl ethers, and a plant-specific blend of volatiles including leaf alcohols are among the numerous products. Thus, the lipoxygenase pathway becomes an initial step in the interaction of plants with pathogens, insects, or abiotic stress and at distinct stages of development (Feussner & Wasternack, 2002).

4.2 In humans

Besides polyunsaturated fatty acids, H_2O_2, fatty acid hydroperoxides, and synthetic organic hydroperoxides support the lipoxygenase-catalyzed xenobiotic oxidation the major reactions documented thus far including oxidation, epoxidation, hydroxylation, sulfoxidation, desulfuration, dearylation, and N-dealkylation (Kulkarni, 2001). It is noteworthy that lipoxygenases are also capable of glutathione conjugation of certain xenobiotics (Kulkarni, 2001). Available data suggest that lipoxygenases contribute to in vivo metabolism of endobiotics and xenobiotics in mammals (Kulkarni, 2001).

Recent reviews describe the role of lipoxygenase in cancer (Bhattacharya et al., 2009; Pidgeon et al., 2007; Moreno, 2009), inflammation (Duroudier et al., 2009; Hersberger, 2010) and vascular biology (Chawengsub et al., 2009; Mochizuki & Kwon, 2008) and for an extensive presentation of the role of eicosanoids in prevention and management of diseases the reader is referred to the review of Szefel et al. (2011).

5. Interaction of lipoxygenase with inhibitors as theoretical approach for food industry and medical applications

In terms of the structure and function, LOXs are unique, because their metal cofactor is a single ion bound by the side chains of the surrounding amino acids and the carboxylic group of the C-terminus, and their inhibitors bind to or near the Fe co-factor (Skrzypczak-Jankun et al., 2007). Lipoxygenases are inhibited by a large number of chemicals, some of which also serve as co-substrates (Kulkarni, 2001).

5.1 Importance of lipoxygenase inhibition for food industry

Besides their physiological role, plant lipoxygenases are of significant importance to the food industry, since these enzymes have been implicated in the generation of the flavour and aroma in many plant products. For instance, they are responsible for the undesirable 'beany', 'green' and 'grassy' flavours produced during processing and storage of protein products derived from legume seeds (Fukushima, 1994; Robinson et al., 1995) and the development of the stale flavour in beer during storage (Kobayashi et al., 1993). Lipoxygenases also play an important role in the baking industry. They are quite effective as bleaching agents, increase mixing tolerance and improve dough rheology (Nicolas & Potus, 1994; Larreta-Garde, 1995; Cumbee et al., 1997; Borelli[b] et al., 1999).

Freshly refined soybean oil is practically odourless and bland, but "green, grassy, fishy" off-flavors may develop quickly if the oil is heated or stored under conditions that expose it to light and oxygen or by contamination with pro-oxidant metals such as copper and iron (Berk, 1992). "Beany" flavour is the principal inconvenience of traditional soymilk and its products (e.g., tofu) and is caused by some ketones and aldehydes, particularly hexanals and heptanals, produced through LOX catalyzed oxidation (Berk, 1992).

Fish lipids are susceptible to oxidation owing to the high levels of polyunsaturated fatty acids (PUFA), even in frozen storage, and this can affect the flavour, texture, taste, aroma and shelf life of fish (Ke & Ackman, 1976). Since the direct interaction between oxygen and highly unsaturated lipids is kinetically hindered (Kanner et al., 1987), the enzymatic initiation of oxidation by enzymes such as lipoxygenase, peroxidases and microsomal enzymes has been gaining favour.

Green tea glazing was shown to improve the storage quality of frozen bonito fillets (Lin & Lin, 2005). In addition, hot water tea extract was shown to suppress the pro-oxidant activities of the dark meat and skin of blue sprat (Seto et al., 2005). Banerjee (2006) proposes that the improvement in the shelf life of fish by green tea polyphenols is at least in part due to inhibition of LOX resulting in delaying oxidation of fish lipids and because of that impregnation of muscle fillets in tea extract by itself or in combination with other natural inhibitors may improve the shelf-life and storage quality of fish fillets.

Besides its function of oxidizing the polyunsaturated fatty acids (linoleic, linolenic and arachidonic), the enzyme may also catalyse the co-oxidation of carotenoids, resulting in the loss of natural colorants and essential nutrients (Robinson et al., 1995). LOX have been

implicated in the generation of the flavour and aroma in many plant products, in the decolourisation of pigments and in the potential of compromising the anti-oxidant status (Casey, 1999). In pasta the involvement of LOX in colour loss is demonstrated by positive correlation between the decrease of β-carotene content after pastification and LOX activities in semolina. In addition to this, the hydroperoxidation and bleaching activities of LOX are highly correlated demonstrating that the bleaching might be ascribable to a co-oxidative action by LOX (Borrelli[a] et al., 1999).
During pasta processing in which the maximal pigment degradation by LOX activity occurs (Borrelli[b] et al., 1999), it is shown that externally added β-carotene can act as inhibitor of the LOX-catalysed linoleate hydroperoxidation and an inverse relation between the % of carotenoid loss and the initial carotenoid content in semolina from durum varieties, showing similar LOX activity, was found (Trono et al., 1999).
The complete characterisation of lipoxygenase from pea seeds (*Pisum sativum var. Telephone L.*) gives possibility to avoid destructive influence during food processing and storage (Szymanowska et al., 2009) by the action of this enzyme.

5.2 LOX inhibition in cancer

Molecular studies of the well-known relationship between polyunsaturated fatty acid metabolism and carcinogenesis have revealed novel molecular targets for cancer chemoprevention and treatment (Lipkin et al., 1999; Willett, 1997; Klurfeld & Bull, 1997; Guthrie & Carroll, 1999).
The role of lipoxygenase in the development and progression of cancer is complex due to the variety of lipoxygenase genes that have been identified in humans, in addition to different profiles of lipoxygenase observed between studies on human tumor biopsies and experimentally induced animal tumor models (Pidgeon et al., 2007). The literature emerging on the role of lipoxygenases in tumor growth, for the most part, suggests that distinct lipoxygenase isoforms, whose expression are lost during the progression of cancer, may exhibit anti-tumor activity, while other isoforms may exert pro-tumorigenic effects and are preferentially expressed during the development of various cancers.
The involvement of 5-lipoxygenase and 12-lipoxygenase in human cancer progression is now supported by a growing body of literature. The involvement of 15-lipoxygenase-1 in colorectal cancer involves its implication in carcinogenesis having pro-carcinogenic as well as anti-carcinogenic roles (Bhattacharya et al., 2009). The co-localization of these enzymes and the similarities of their bioactions on cancer cell growth suggest that the simultaneous inhibition of these enzymes may represent novel and promising therapeutic approaches in selected cancer types (Pidgeon et al., 2007). Therefore, when targeting the regulation of arachidonic acid metabolism, blocking 5-lipoxygenase, 12-lipoxygenase and 15-lipoxygenase-1 without altering the expression of the anti-carcinogenic 15-lipoxygenase-2 may be the most effective, however at present no drug recapitulates these capabilities (Pidgeon et al., 2007).

5.3 Mechanisms of lipoxygenase inhibition

In general, lipoxygenase inhibitors can bind covalently to iron or form the molecular complexes blocking access to iron (Skrzypczak-Jankun et al., 2007). It was pointed out by Walther et al., that a course of inhibition, by the drug ebselen, (noncompetitive vs competitive) and its reversibility depend on the oxidation state of iron, i.e. whether the enzyme is catalytically silent with Fe^{2+} when it binds covalently, causing irreversible

inhibition or preoxidized and active with Fe^{3+} in the presence of the fatty acid substrate (Walther et al., 1999). In both cases the enzyme's performance can be illustrated by a classic Lineweaver-Burk plot. Many inhibitors do not follow such a linear relation between velocity and the inhibitor's concentration showing a hyperbolic curve instead as observed by Skrzypczak-Jankun et al. (2002) for polyphenolic inhibitors (curcumin, quercetin, epigallocatechin gallate and epigallocatechin) interacting with soybean lipoxygenase-3. In general, the kinetic data are seldom reported (Skrzypczak-Jankun et al., 2007). Xenobiotic oxidation by soy lipoxygenase has been investigated and described, while human enzymes lack such thorough studies (Skrzypczak-Jankun et al., 2007). The in vivo susceptibility of lipoxygenases' inhibitors may depend not only on the source of lipoxygenase and its isozyme (Pham et al., 1998; Schewe et al., 1986) but also on the oxidation state of iron and the competition between peroxidase and co-oxidase activities of enzyme (Borbulevych et al., 2004).

The first mode to inhibit the lipoxygenase would be a direct reduction of iron to its inactive form. For soybean lipoxygenase, it has been demonstrated that nordihydroguaiaretic acid rapidly reduces the active ferric species of the enzyme to its inactive ferrous form, thus causing interruption of the catalytic cycle (Kemal et al., 1987). For the polyphenol inhibition of lipoxygenase it was firstly suggested that this molecules strongly complex of the ferric iron moiety of the lipoxygenase, thus preventing its reduction *via* the catalytic cycle as proposed for the action of 4-nitrocatechol on the soybean lipoxygenase-1 (Spaapen et al., 1980). The second observation that complexation of the flavanols with Fe^{3+} did not abolish the inhibitory effect may rule out a direct complexation of the iron moiety in ferric lipoxygenase by these catechol compounds. The X-ray analysis shows 4-nitrocatechol near iron with partial occupancy, blocking access to Fe but not covalently bound to it (Skrzypczak-Jankun et al., 2004). If a similar mode of action holds for the interaction of flavanols with mammalian lipoxygenases, the corresponding iron polyphenol complexes may retain their lipoxygenase-inhibitory effect.

A third conceivable mode of action of polyphenols is the effective reduction of hydroperoxides that are essential activators of lipoxygenase *via* conversion of the enzymatically silent ferrous species to the active ferric form. The observation of Schewe et al. (2001) that lowering of the hydroperoxide tone by glutathione plus glutathione peroxidase did not modulate the inhibitory effects of flavanols on 15-lipoxygenase-1 does not support the latter possibility.

In case of carotenoids, more specifically, β-carotene, the lipoxygenase was inhibited by keeping it in the inactive form of Fe(II) (Serpen & Gökmen, 2006). These authors suggest that β-carotene reacts with linoleyl radical (L•) at the beginning of the chain reaction, so it prevents the accumulation of conjugated diene forms (LOO•, LOO− and LOOH). Since L• transforms back to its original form of LH, the enzyme cannot complete the chain reaction and thus remains in the inactive Fe(II) form, which is not capable of catalyzing linoleic acid hydroperoxidation (Serpen & Gökmen, 2006).

Wu et al. (1999) have reported that β-carotene scavenges the linoleyl peroxy radical (LOO•) by a hydrogen transfer mechanism and the oxidation of β-carotene occurs during this action. In these conditions, it is absolutely clear that the amount of inactivated enzyme depends on the concentration of β-carotene present in the medium (Serpen & Gökmen, 2006).

According to Mahesha et al. (2007) the lipoxygenase inhibition by isoflavones follows the next mechanism: an electron donated by isoflavones is accepted by the ferric form (Fe^{3+}) of lipoxygenase, which is reduced to resting ferrous form (Fe^{2+}), thus inhibiting lipoxygenase.

Genistein is neither consumed nor does state change during the course of the reaction of lipoxygenase (Mahesha et al., 2007), while quercetin entrapped within lipoxygenase undergoes degradation (Borbulevych et al., 2004).

5.4 Inhibition of soybean lipoxygenase by different classes of polyphenols

Gillmor et al. (1997) obtained the structure of the rabbit reticulocyte enzyme as a complex with the inhibitor RS75091. Located in one of the hydrophobic channels of the enzyme, the inhibitor was found to be close but not biding the iron atom of the catalytic situs. These observations provided the first indications of how the native enzyme can interact with potential ligands (Pham et al., 1998).

Natural flavonoids don't affect only the lipoxygenase oxidation of its classical substrates but also the co-oxidation of xenobiotics by this enzyme. Epigallocatechin-gallate, quercetin and rutin proved to reduce the co-oxidation rate of guaiacol, benzidine, paraphenylenediamine and dimethoxybenzidine by soybean lipoxygenase-1 (Hu et al., 2006). This data suggest that flavonoids may have anticarcinogenic and antitoxic effect through inhibition of oxidative activation generated by lipoxygenase (Hu et al., 2006). Green tea polyphenols have potent free radical quenching and antioxidant activities (Wiseman et al., 1997) and have structural features that may specifically interfere with the arachidonic acid cascade, including the lipoxygenase pathway (Hong & Yang, 2003; Hussain et al., 2005). In addition, with growing concerns regarding the safety of synthetic antioxidants such as BHT and BHA, alternative mechanisms of antioxidant protection by the use of natural antioxidants have been in review over the years (Barlow, 1990).

Polyphenols, mainly flavonoids and phenolic acids, are abundant in a number of dietary sources such as certain cocoas, tea, wine, fruits and vegetables. More than 8000 different flavonoids of natural origin are known (Schewe & Sies, 2003). The flavonoids exist in nature as aglycons (free form) or conjugated (with O-glucosides or methylated). The aglycons can be subdivided in different subclasses (flavanols, flavanones, flavones, izoflavones, flavonols, anthocyanidines, aurones, chalcones) in function of how the B ring from their structure is linked to the heterocycle C, of the oxidation state and of the functional groups linked to the C ring (Beecher, 2003).

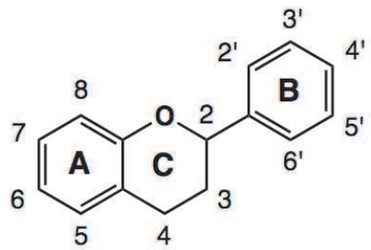


Fig. 3. The basic structure of flavonoids.

The basic structure of flavonoids is represented by the flavan nucleus containing 15 carbons structured in 2 benzene rings, named A and B and linked by a C_3 unit, which together with an oxygen atom forms the γ-pyronic or γ-pyranic ring, named the C ring as shown in Fig.3.

A number of *in vitro* and *in vivo* studies as well as clinical trials suggest beneficial effects of flavonoids for health, counteracting the development of cardiovascular diseases, cancer and obesity. Bors et al. (1990) were the first to claim three partial structures contributing to the

radical-scavenging activity of flavonoids: (a) an o-dihydroxyl structure in the B ring (catechol structure) as a radical target site providing good electron delocalization and stabilization of the phenoxy radical; (b) a 2,3-double bond with conjugation to the 4-oxo group which is necessary for delocalization of an unpaired electron from the B ring, (c) hydroxyl groups at the 3-and 5-positions, which are necessary for enhancement of radical scavenging activity, increasing the delocalisation of electrons across the flavonoid scaffold. The catechol group is essential for the radical-scavenging activity of flavan-3-ols and flavanones lacking 2,3-double bonds (Bors et al., 1990).

In parallel to the free radical-scavenging properties the following structural features were found to enhance the inhibitory potency: (i) presence of a catechol arrangement in the B or A ring, (ii) a carbonyl group together with a 2,3-double bond in the C ring (Schewe & Sies, 2003). Other structural features were opposite to the free radical scavenging potencies: (i) presence of a 3-OH group in the C ring diminished than reinforced the inhibition of lipoxygenases, (ii) in the absence of a catechol arrangement there was an inverse correlation to the total number of OH groups in the flavonoid molecule (Schewe & Sies, 2003). Although either reducing or ferric iron chelating properties are prerequisites for a lipoxygenase- inhibitory compound, and both of them are also inherent to flavonoids, the inhibitory effects cannot be ascribed solely to one of these mechanisms (Schewe & Sies, 2003). The inhibition of lipoxygenases by flavonoids appears to be of more complex nature (Schewe & Sies, 2003).

Inhibitors studies of lipoxygenase from pea showed that phenolic antioxidant components were effective and can be used to protect food lipids against oxidation (Szymanowska et al., 2009). The conducted research proved that activity of lipoxygenase from pea seeds could be effectively inhibit by some phenolic compounds. The most effective inhibitor is caffeic acid (about 57% of inhibition). Flavonoids like catechin and quercetin considerably inhibit the lipoxygenase activity. Inhibitors used for investigation in this study were placed in the following order: caffeic acid > quercetin > catechin > benzoic acid > ferulic acid > kaempferol (Szymanowska et al., 2009).

5.5 Lipoxygenase inhibition by quercetin

Quercetin is the most abundant among the flavonoid molecules and can be found in the fruits, vegetables, seeds, nuts, and flowers of many plants. Its documented impact on human health includes cardiovascular protection, anticancer, antiviral, anti-inflammatory activities, antiulcer effects and cataract prevention. Like other flavonoids, quercetin appears to combine both lipoxygenase-inhibitory activities and free radical-scavenging properties in one agent and thus belongs to a family of very effective natural antioxidants (Sadik et al., 2003). Quercetin is a flavonol that can be easily oxidized in an aqueous environment, and in the presence of iron and hydroxyl free radicals (Borbulevych et al., 2004).

The inhibition of rabbit 15-lipoxygenase-1 and of soybean lipoxygenase-1 by quercetin was studied in detail (Sadik et al., 2003). Quercetin modulates the time course of the lipoxygenase reaction in a complex manner by exerting three distinct effects: (i) prolongation of the kinetic lag period, (ii) instant decrease in the initial rate after the lag phase being overcome, (iii) time-dependent inactivation of the enzyme during reaction, but not in the absence of substrate (Schewe & Sies, 2003). The literature data obviously indicate that quercetin represents one of the most potent inhibitors of different LOXs (Schneider & Bucar[a], 2005; Schneider & Bucar[b], 2005).

Structural analysis reveals that quercetin entrapped within LOX undergoes degradation and the resulting compound has been identified by X-ray analysis as protocatechuic acid (3,4-dihydroxybenzoic acid) positioned near the iron site (Borbulevych et al., 2004).

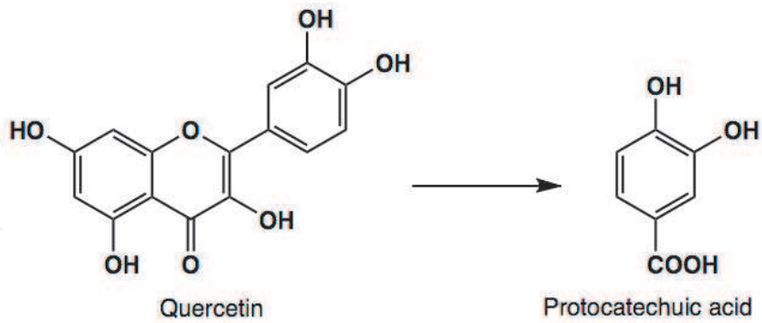


Fig. 4. Product of quercetin degradation by soybean LOX-3 (Borbulevych et al., 2004).

We demonstrated that pH values may influence the molecular interactions between soybean LOX-1 and quercetin, and especially the alcaline pH favours the ionic display of quercetin in order to interact with LOX better (Chedea et al., 2006).
Quercetin inhibited the 12 (S)-hydroxytetraenoic acid production at concentrations below those necessary for growth inhibition in colorectal cancer cells overexpressing the enzyme 12(S)-lipoxygenase with an IC_{50} of 1μM (Bednar et al., 2007). The finding that LOX can turn different compounds into simple catechol derivatives (with one aromatic ring only) might be of importance as an additional small piece of a "jigsaw puzzle" in the much bigger picture of drug metabolism (Borbulevych et al., 2004). Their interactions with LOX can be more complicated than simply blocking the access to the enzyme's active site. The studies on LOX and quercetin contribute to the understanding of biocatalytic properties of this enzyme and its role in the metabolism of this popular (as a medicinal remedy) flavonol and possibly other, similar compounds (Borbulevych et al., 2004). Acting both as a substrate and a source of inhibition, quercetin seems to play an antinomic role (Fiorucci et al., 2008). But this could be explained as quercetin, one of the most representative flavonoids, is a highly functionalized substrate and can thus be activated and degraded following several ways (Fiorucci et al., 2008).

5.6 Inhibition of soybean lipoxygenase by epigallocatechin gallate

Flavanols (or flavan-3-ols or catechins) are a class of flavonoids that include the catechins and the catechin gallates. Catechins are described as colorless, astringent, water-soluble polyphenols found in many fruits and grains, such as coffee, red grapes, prunes and raisins. Their main source however comes from a beverage made from tea leaves of *Camellia sinensis*. (-)-Epigallocatechin gallate (EGCG) together with other galloylated catechins constitute more than 90% of the total catechin content in green tea (Lekli et al., 2010). Laboratory studies strongly indicate that tea inhibits certain cancers, and there is a multitude of evidence confirming the antioncogenic properties of the individual catechins. For instance: EGCG alone shows anticancer effectiveness against carcinogen-induced skin, lung, forestomach, esophagus, duodeum, liver and colon tumors in rodents. It was found to cause apoptosis and/or cell cycle arrest in human carcinoma cells of skin and prostate cancers (Zimeri & Tong, 1999). Catechins have also known inhibitory activity toward dioxygenases with a potential to be utilized in disease prevention and treatment.

The study of Banerjee (2006) shows that green tea polyphenols are very potent inhibitors of mackerel muscle LOX, with EGCG (epigallocatechin gallate) as the most effective inhibitor (IC_{50} 0.13 nM) followed by ECG (epicatechin gallate) (IC_{50} 0.8 nM), EC (epicatechin) (IC_{50} 6.0 nM), EGC (epigallocatechin) (IC_{50} 9.0 nM) and C (catechin) (IC_{50} 22.4 nM). Chocolate and cocoa are also sources of catechins. Epigallocatechin gallate isolated from the seeds of *Theobroma cacao* had the best inhibitory activity on rabit 15-lipoxygenase-1, with an IC_{50}=4M, epicatechin gallate had IC_{50}=5M and epicatechin an IC_{50}=60M (Schewe et al., 2002). A better inhibition of epicatechin (IC_{50} approx. 15M) was registered in the case of recombinant human platelet 12-lipoxygenase.

Obtained from X-ray analysis, the 3D structure of the resulting complex of (-)-epigallocatechin gallate (EGCG) interacting with soybean lipoxygenase-3 reveals the inhibitor depicting (-)-epigallocatechin that lacks the galloyl moiety (Skrzypczak-Jankun et al., 2003). The A-ring is near the iron co-factor, attached by the hydrogen bond to the C-terminus of the enzyme, and the B-ring hydroxyl groups participate in the hydrogen bonds and the van der Waals interactions formed by the surrounding amino acids and water molecules (Skrzypczak-Jancun et al., 2003).

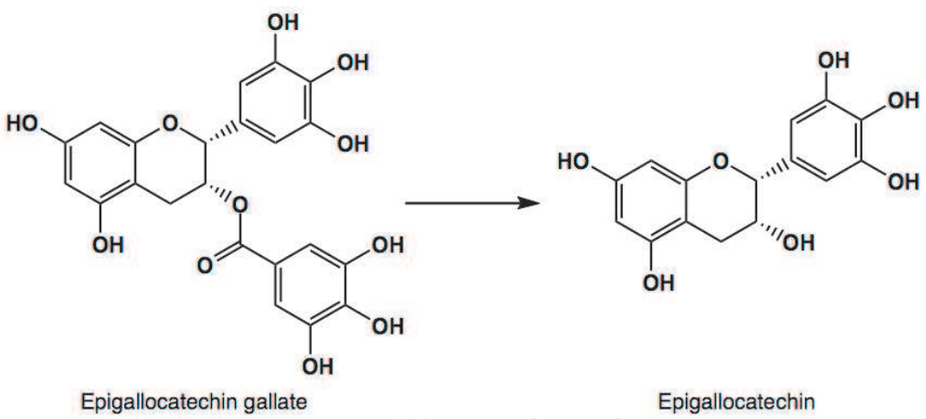


Fig. 5. Lipoxygenase-3 in complex with epigallocatechin gallate as an inhibitor determines the degradation of natural flavonoid to epigallocatechin (Skrzypczak-Jancun et al., 2003).

X-ray analysis of soybean lipoxygenase-3 crystals soaked with EGCG shows the molecular complex of LOX-3 with (-)epigallocatechin molecule.

5.7 Inhibitory effects of soybean isoflavones on lipoxygenase activity

Soybeans are important sources of isoflavone levels (Song et al., 1998), present as 12 derivatives, including free genistin, daidzin, glycitin and their acetyl, malonyl or glycosilated forms. Isoflavones are composed of 2 benzene rings (A and B) linked through a heterocyclic pyrane C ring. The position of the B ring discriminate flavonoid flavones (C2-position) from isoflavones (C3-position).

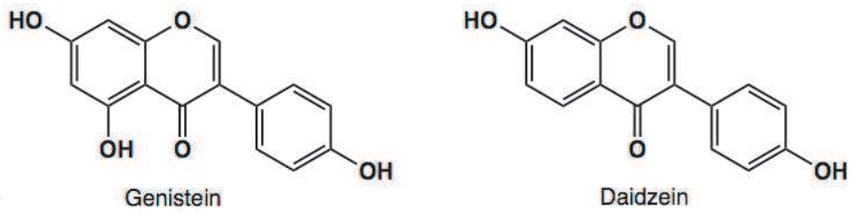


Fig. 6. Dietary isoflavones inhibitors of lipoxygenase.

The impact of dietary isoflavones, daidzein and genistein, on the health of adults and infants is well documented, an increasing interest for these compounds being registered due to their biological effects including: estrogen-like activity, prevention of breast (Warri et al., 2008), prostate (Matsumura et al., 2008) and colon cancer (Mac Donald et al., 2005), antioxidant activity (Malencić et al., 2007; Sakthivelu et al., 2008), prevention of menopausal symptoms and osteoporosis (Ma et al., 2008), and heart disease (Xiao, 2008).
In the work of Mahesha et al. (2007) the inhibition of soy lipoxygenase-1 and 5-lipoxygenase from human polymorph nuclear lymphocyte by isoflavones, genistein and daidzein as glycosilated and unglycosilated compounds was studied. Soybean isoflavones inhibit LOX either as aglycons, or as glucosides. Isoflavones exert combined dual actions as inhibitors: they compete with the hydroperoxide formation to prevent the generation of LOX active ferric state (1) and also are capable of reducing the ferric enzyme to its inactive ferrous form (2) (Mahesha et al., 2007).
Vicaş et al. (2011) showed that genistein was almost twice more potent inhibitor than daidzein at similar concentration with concentration that induces 50% soybean lipoxygenase-1 inhibition values of 5.33 mM versus 11.53 mM. Genistein and daidzein proved to be noncompetitive with inhibition constants K_i of 33.65 and 43.45 mM, respectively (Vicaş et al., 2011). The inhibitory efficiency of the genistein and daidzein depended both on their concentration and on the substrate's concentration (Vicaş et al., 2011).

5.8 Inhibition of soybean lipoxygenase by carotenoids

There are over 600 fully characterized, naturally occurring molecular species belonging to the class of carotenoids. In humans, some carotenoids (the provitamin A carotenoids: α-carotene β-carotene, γ-carotene and the xanthophyll, β-cryptoxanthin) are best known for converting enzymatically into vitamin A; diseases resulting from vitamin A deficiency remain among the most significant nutritional challenges worldwide. Also, the role that carotenoids play in protecting those tissues that are the most heavily exposed to light (e.g. photo protection of the skin, protection of the central retina) is perhaps most evident, while other potential roles for carotenoids in the prevention of chronic diseases (cancer, cardiovascular disease) are still being investigated. Because carotenoids are widely consumed and their consumption is a modifiable health behaviour (via diets or supplements), health benefits for chronic disease prevention, if real, could be very significant for public health (Mayne, 2010). Carotenoids are isoprenoid molecules which contain a polyene chains, with or without cyclisation at the ends.

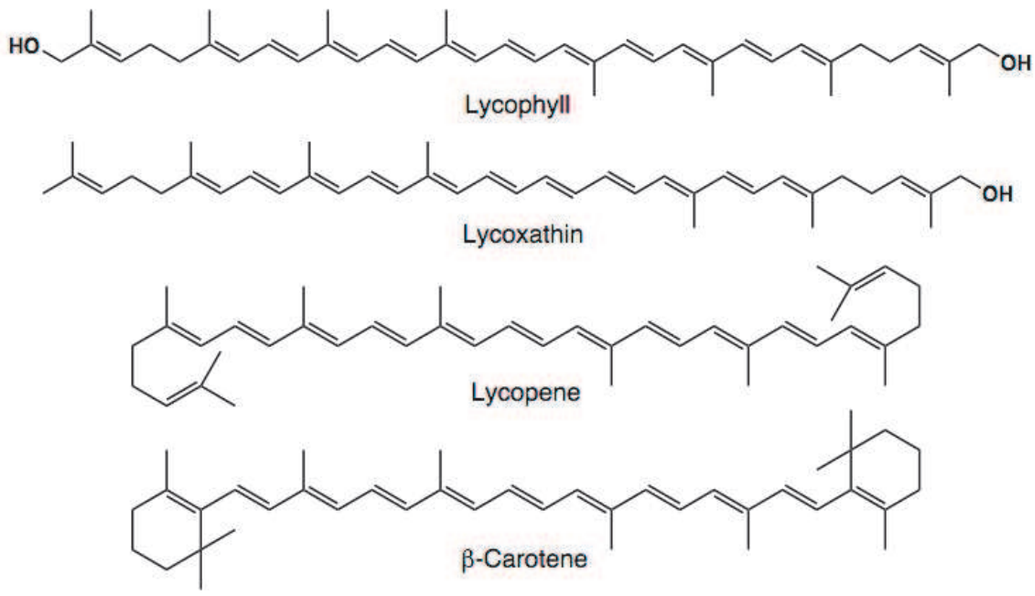


Fig. 7. Carotenoids serving as lipoxygenase co-substrates

The existence of an enzyme "carotene oxidase" in soybeans, which catalyzes the oxidative destruction of carotene was reported by Bohn and Haas in 1928 (Bohn & Haas, 1928). Four years later, Andre and Hou found that soybeans contained an enzyme, lipoxygenase (linoleate oxygen oxidoreductase), which they termed "*lipoxidase*", catalyzing the peroxidation of certain unsaturated fatty acids (Andre & Hou, 1932).

In 1940 the observation that "lipoxydase" is identical to "carotene oxidase" was published (Sumner & Sumner, 1940). These early findings of lipoxygenase peroxidizing the unsaturated fats and bleaches the carotene were reported as the result of studies on the oxidation of crystalline carotene or carotene dissolved in unsaturated oil. Surprisingly it was found that the carotene oxidase had an almost negligible bleaching action upon the crystalline carotene. On the contrary, when one employs carotene dissolved in a small quantity of fat, the bleaching is extremely rapid. With excessive quantities of fat, the rate of bleaching of the carotene diminishes, and it was concluded that the effect of added fat upon the rate of bleaching of carotene is probably due to a coupled oxidation (Sumner & Sumner, 1940).

Studying the soya-lipoxygenase-catalyzed degradation of carotenoids from tomato Biacs and Daood (2000) found that β-carotene was the most sensitive component, followed by lycoxanthin and lycopene. Their results also implied that β-carotene can actively perform its antioxidant function during the course of lipid oxidation. It seems that oxidative degradation and, accordingly, antioxidant activity of each carotenoid depends on the rate of its interaction with the peroxyl radical produced through the lipoxygenase pathway (Biacs & Daood, 2000) and thus is able to inhibit lipoxygenase. The inhibition of the hydroperoxide formation by carotenoids has been attributed to their lipid peroxyl radical-trapping ability (Burton & Ingold, 1984).

In vitro, lycopene is a substrate of soybean lipoxygenase. The presence of this enzyme also significantly increased the production of lycopene oxidative metabolites (dos Anjos Ferreira et al., 2004; Biacs & Daood, 2000). It was reported that during the co-oxidation of β-carotene

by LOX-mediated hydroperoxidation reactions, inhibition of LOX activity takes place also (Lomnitski et al., 1993; Trono et al., 1999; Pastore et al., 2000). The activity of soybean lipoxygenase-1 was inhibited by β-carotene which breaks the chain reaction at the beginning stage of linoleic acid hydroperoxidation (Serpen & Gökmen, 2006). Besides soybean lipoxygenase (Ikedioby & Snyder, 1977; Hildebrand & Hymowitz, 1982) carotene oxidation during lipoxygenase-mediated linoleic acid oxidation has been reported in various studies for the enzymes extracted from potato (Aziz et al., 1999), pea (Yoon & Klein, 1979; Gökmen et al., 2002), wheat (Pastore et al., 2000), olive (Jaren-Galan et al., 1999) and pepper (Jaren-Galan & Minguez-Mosquera, 1997). Soybean lipoxygenase-1 and recombinant pea lipoxygenase-2 and lipoxygenase-3, oxidizing β-carotene, yield apocarotenal, epoxycarotenal, apocarotenone and epoxycarotenone (Wu et al., 1999).
Through molecular modeling Hazai et al. (2006) predicted that lycopene and lycophyll bind with high affinity in the superficial cleft at the interface of the β-barrel and the catalytic domain of 5-LOX (the "cleavage site") suggesting potential direct competitive inhibition of 5-LOX activity by these molecules after in vivo supplementation, particularly in the case of the dial metabolite.

5.9 Quinone and semiquinone formation during the lipoxygenase inhibition reaction

In his excellent review from 2001, Kulkarni presents the studies up to that date indicating the semiquinone and quinone formation in different lipoxygenase catalyzed reactions of xenobiotics oxidation. Diethylstilbestrol (DES) is a human transplacental carcinogen. DES-quinone, one of the metabolites of DES, binds to DNA and is presumed to be the ultimate toxicant. Although DES-quinone formation by human tissue lipoxygenase has yet to be examined, soybean lipoxygenase has been shown to initiate one-electron oxidation to DES semiquinone in the presence of H_2O_2 (Nunez-Delicado et al., 1997). Subsequent dismutation of two molecules of DES semiquinone yields one molecule each of DES-quinone and DES.
Although phenol is oxidized slowly by different lipoxygenase isoenzymes, potato 5-lipoxygenase (Cucurou et al., 1991) and soybean lipoxygenase-1 (Cucurou et al., 1991; Mansuy et al., 1988), substituted phenols and catechols undergo extensive one-electron oxidation and yield the corresponding reactive phenoxyl radicals or semiquinones. These free radicals polymerize to yield a mixture of complex metabolites.
However, it seems that quercetin may act, in most of cases, after being metabolically activated (Metodiewa et al., 1999), and despite a constant increase of knowledge on both positive and negative biological effects of this natural product, it remains often unclear which activated form should play a role in a given process (Fiorucci et al., 2007). Indeed, semiquinone and quinone forms of quercetin, deriving from the abstraction of respectively one or two H•, are involved in many oxidative processes (Metodiewa et al., 1999; Gliszczynska-Swiglo et al., 2003; Hirakawa et al., 2002). For instance, quercetin reduces peroxyl radicals involved in lipid peroxidation, and through this reaction, a semiquinone species is produced, which then undergoes a disproportionation to generate a quinone form (Fiorucci et al., 2007). Three semiquinone forms for quercetin have been considered by Fiorucci et al. (2008) in order to study the quercetin binding to lipoxygenase-3 by molecular modeling simulations. In the case of lipoxygenase–catechol complexes, the formation of the catechol-iron(III) complex of soybean lipoxygenase 1 gradually results in reduction of the cofactor and release of the semiquinone but no evidence of quinone formation in the UV-visible spectra of samples of the native enzyme treated with catechol was obtained (Spaapen et al., 1980; Nelson, 1988; Pham et al., 1998).

Besides their antioxidant properties, catechins have been described to display pro-oxidant activity having the potential to oxidize the quinones or semiquinones resulting in redox cycling and reactive oxygen species production as well as in thiol, DNA and protein alkylation (Galati & O'Brien, 2004; van der Woude et al., 2006).
Our previous study shows that the oxidation products of catechins are formed within the cellular matrix but also in the extracellular medium (Chedea et al., 2010). We have demonstrated by UV-Vis spectroscopy, that the quinones are involved in the modulation of lipoxygenase activity in the presence of catechins within the cells (Chedea et al., 2010). This conclusion is in agreement with that of Sadik et al. (2003) and Banerjee (2006). An irreversible covalent modification of soybean LOX by flavonoids has been suggested by Sadik et al. (2003) whereby during the formation of fatty acid peroxyl radical in the LOX pathway, the flavonoids are co-oxidized to a semi-quinone or quinone, which in turn may bind to sulfhydryl or amino groups of the enzyme causing inhibition (Banerjee, 2006).

6. Conclusions

The knowledge presented in this study addressed the lipoxygenase pathway inhibition by antioxidant polyphenols at two levels: human diet and human health or to a larger extent human disease prevention and treatment. Review articles (Jachak, 2006; Schneider & Bucar[a], 2005; Schneider & Bucar[b], 2005) summarize natural products with inhibitory properties toward LOX (Skrzypczak-Jankun et al., 2007). Natural remedies almost never consist of a single ingredient and usually are a mixture of many in proper proportions, with a synergistic effect of their simultaneous action being absolutely necessary for beneficial medicinal results. Thus, one should proceed with caution, since the action of a selected single compound may not be the same (Skrzypczak-Jankun et al., 2007). The X-ray studies of soybean complexes with quercetin, curcumin, EGCG, EGC indicated conversion of these inhibitors into their metabolites (Skrzypczak-Jankun et al., 2007), which is not surprising considering the co-oxidative activity of LOXs (Kulkarni, 2001). As already presented a question arises concerning the lipoxygenase inhibion: "What is really inhibiting LOX, a given chemical or its LOX metabolite?" (Skrzypczak-Jankun et al., 2007). The results presented so far indicate a complex mode of inhibition involving the inhibitor itself but also its reaction product with lipoxygenase.
The lipoxygenase researcher faces the next antinomy: despite the difference in the number of amino acids between plant and mammalian LOXs, these proteins are amazingly similar in topology with high similarities in the active site of these enzymes. It is believed that all LOXs follow the same catalytic mechanism; however, it is probably the vicinity of the iron site that determines the regio and stereospecificity of the particular enzyme (Skrzypczak-Jankun et al., 2003). In this contradictorily state of facts soybean lipoxygenases stands as a control point in terms of structure, activity and thus inhibition.

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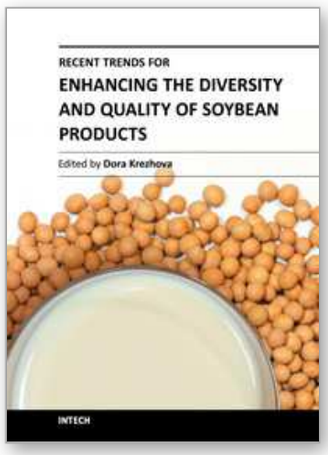
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This book presents new aspects and technologies for the applicability of soybean and soybean products in industry (human food, livestock feed, oil and biodiesel production, textile, medicine) as well as for future uses of some soybean sub-products. The contributions are organized in two sections considering soybean in aspects of food, nutrition and health and modern processing technologies. Each of the sections covers a wide range of topics. The authors are from many countries all over the world and this clearly shows that the soybean research and applications are of global significance.

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